Vol. LXIV, No. 3 June, 1933

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

HEMOLYTIC ACTION OF SILVER OCCURRING AS AN IMPURITY IN CHEMICALLY PURE SODIUM CHLORIDE[1]

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The hemolysis of fish erythrocytes when placed in isotonic solutions made from certain brands of sodium chloride C.P. has been reported by Williams and Jacobs (1931). The experimental results presented by these investigators suggest an impurity in the salt as the causative agent. Since sodium chloride is the main constituent of most isotonic mediums used in biological experiments it seemed of importance to determine the exact nature of this toxic principle. Data will be presented in this paper to show that the hemolysis of fish erythrocytes by isotonic solutions made from certain brands of so-called chemically pure sodium chloride is due to the presence of small amounts of silver occurring as an impurity in the salt.

Procedure

Fish were bled directly from the ventral aorta into a beaker. The blood was defibrinated and the fibrin removed by filtering through dry cheesecloth that had been washed in distilled water. The blood was then centrifuged for five minutes at a rate just sufficient to cause separation of the cells and serum and the cell volume roughly adjusted when necessary to the normal value of 25 per cent so as to insure uniformity in the cell content of blood obtained from different fish. This procedure was found to be necessary as the rate of hemolysis was affected by the amount of red cells used. One drop of the remixed blood was added from a bulb pipette, constructed so as to deliver uniform drops (1 cc. = 28 drops), to 25 ml. of the isotonic (0.25 M) sodium chloride solutions contained in test tubes (200 × 30 mm.). The progress of hemolysis with time was followed by visual inspection.

[1] Presented before the American Society of Biological Chemists at Philadelphia, April 28–30, 1932.

[2] With the coöperation of Harold Blumberg, Department of Biochemistry, Johns Hopkins School of Hygiene and Public Health.

Results

The blood cells of different species of fish show a wide variation in their resistance to hemolysis when placed in isotonic solutions of various brands of sodium chloride. In Table I are assembled representative times for complete hemolysis of the blood cells of a number of species of marine fish when placed in isotonic solutions made from four different brands of sodium chloride. The blood cells of both the

Table I

Hemolysis time for the erythrocytes of various species in isotonic sodium chloride

Species	Brand 1	Brand 2	Brand 3	Brand 6
Cunner (*Tautogolabrus adspersus*)	No hemolysis	5 min.	6 min.	7 min.
Tinker Mackerel (*Scomber scombrus*)	No hemolysis	5.5 min.	7.5 min.	27 min.
Bull's Eye Mackerel (*Scomber colias*)	No hemolysis	3 min.	4 min.	5 min.
Butterfish (*Poronotus triacanthus*)	No hemolysis	4 min.	5 min.	10 min.
Flounder (*Platessa dentatus*)	No hemolysis	10 min.	10 min.	15 min.
Scup (*Stenotomus chrysops*)	No hemolysis	18 min.	32 min.	
Tautog (*Tautoga onitis*)	No hemolysis	22 min.	34 min.	
Common Sea Robin (*Prionotus carolinus*)	No hemolysis	25 min.	25 min.	30 min.
Rudder Fish (*Palinurichthys perciformis*)	No hemolysis	25 min.	25 min.	20 min.
Dogfish (*Mustelus canis*)	No hemolysis	75 min.	60 min.	

mackerel and the cunner are rapidly hemolyzed. Since both of these species were easily obtainable and afforded a rapid means of testing for the toxic principle they were used throughout in the work that follows. (Table VI excepted.)

Of the four brands shown in Table I only No. 1 was non-toxic. This is a Kahlbaum's salt, which agrees with the findings of Williams and Jacobs. This salt is the only one which is not mined. It is prepared from sodium carbonate and hydrochloric acid in porcelain vats. This fact would immediately lead one to suspect the occurrence of an

impurity in the other salts. However, when seeking the cause for toxic actions of single salt solutions on biological material it is always necessary to keep in mind the action of so-called antagonistic ions. That the lack of antagonistic ions cannot be the explanation in this case is shown by the results presented in Table II.

An isotonic solution of sodium chloride which showed rapid hemolysis was progressively diluted with sea water that had also been made isotonic by dilution and which caused no hemolysis. Since sea water contains a suitable mixture of the ions deemed necessary to form a physiologically balanced solution, such admixture should result in the

TABLE II

Effect of dilution of toxic salt solution with isotonic sea water.
0 = no hemolysis; ++++ = complete hemolysis.

Isotonic* sea water	0 ml.	5 ml.	10 ml.	15 ml.	20 ml.	25 ml.
NaCl No. 2	25 ml.	20 ml.	15 ml.	10 ml.	5 ml.	0 ml.
Hemolysis Time *min.*						
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	++	+	0	0	0	0
5	++++	+	0	0	0	0
6		++	+	0	0	0
7		++++	++	0	0	0
8			++	0	0	0
9			+++	+	0	0
10			++++	++	0	0
15				++++	0	0
100					++++	0

* Similar results obtained when a non-toxic salt solution substituted for isotonic sea water.

complete disappearance of the toxic action. However, as can be seen from the data presented, the toxic effect persisted, though diminished, even when sea water formed as much as 80 per cent of the mixture. Moreover, almost identical results were obtained when the dilution was made with a non-toxic sodium chloride solution. Thus the presence of calcium, magnesium, and potassium had no effect on the toxic action. Therefore, it seemed reasonable to conclude that the toxic action was caused by an impurity.

In order to determine whether this impurity present in sodium chloride was organic or inorganic, samples of salts that showed hemolytic effects were fused in a platinum crucible and held in the liquid

state for at least fifteen minutes. Such treatment should destroy, at least partially, any organic matter present. Hence if the impurity is organic in nature, isotonic solutions made from the fused salt should cause little or no hemolysis whereas the toxic action should remain undiminished if the impurity is inorganic.

As shown in Table III, fusing caused no diminution in the toxic action of a salt; in fact, an increase in toxicity seemed to have occurred. Hence the impurity was classed as an inorganic substance. The only reasonable explanation for the increased toxicity after fusion seemed to be the formation of oxides with an attendant increase in alkalinity of the resulting solution. Rough tests with indicators showed that

TABLE III

Hemolytic action of various salts before and after fusion.

S.G. = swirl gone—disappearance of characteristic swirl shown by cells on shaking.

Salt*	1	1 Fused	2	2 Fused	3	3 Fused	4	5	6
Hemolysis Time *min.*									
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	S.G.	++	0	++	0	0	0
4	0	0	++	++++	S.G.	++++	0	0	0
5	0	0	++++		S.G.		0	0	S.G.
6	0	0			+		0	0	+
7	0	0			++		0	0	+++
8	0	0			+++		0	0	++++
9	0	0			++++		0	0	
10	0	0					0	0	
300	0	0					0	0	

* Each number represents a different brand of sodium chloride, except No. 5 which was prepared from recrystallized sodium carbonate and hydrochloric acid.

such was the case, suggesting that the toxic action might vary with pH.

In the hope that some light might be thrown on the nature of the inorganic impurity present in the sodium chloride, a study of the effect of pH on the rate of hemolysis was made. The results of one such experiment are shown in Table IV. The pH was controlled by adding 2 ml. of buffer to 23 ml. of isotonic salt solution, a procedure which altered the isotonicity but little. The difference in pH produced by dilution of the buffer was not detectable colorimetrically. As the pH increases, the rate of hemolysis also increases. Below pH 5.0 the hemolytic action is very slow and if present is masked by a change produced on the cells by the acid solutions. This effect of pH sug-

TABLE IV

Effect of pH on Hemolysis Time.

pH	3.0		4.0		5.0		6.0		7.0		8.0		9.0		10.0	
Salt*	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Hemolysis Time *min.*																
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0	++
3	0	0	0	0	0	0	0	0	0	+	0	+++	0	++++	0	++++
4	0	0	0	0	0	0	0	0	0	+++	0	++++	0		0	
5	0	0	0	0	0	0	0	+	0	++++	0		0		0	
10	0	0	0	0	0	+	0	++++	0		0		0		0	
15	0	0	0	0	0	++++	0		0		0		0		0	
30	†	†	†	†	0		0		0		0		0		0	

* All toxic salts showed this pH effect.

† Cells appear to have undergone a change, though not hemolysis.

gested as a working hypothesis, since the impurity was inorganic, that the causative agent was a cation combining with the protein of the cell wall only on the alkaline side of its isoelectric point. That this viewpoint of the mechanism may be entirely erroneous is fully recognized, but as a working hypothesis in the identification of the impurity it proved useful.

It now seemed reasonable to suppose that since all the brands of sodium chloride that contained this impurity came from a natural source, the unpurified or table salt would contain larger quantities of the toxic inorganic substance. Isotonic solutions made from several samples of table salt were found to be turbid because of the ingredients added to prevent caking. On filtering these solutions, it was found

TABLE V

Removal of Impurity by Adsorption on Activated Charcoal and Recovery on Ashing

Salt	1	1N*	2	2N	3	3N	6	6N	1A†	1B‡
Hemolysis Time *min.*										
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	+	0	0	0
4	0	0	++	0	+	0	++	0	0	0
5	0	0	++++	0	++	0	+++	0	S.G.	0
6	0	0		0	+++	0	++++	0	S.G.	0
7	0	0		0	++++	0		0	+	0
10	0	0		0		0		0	++++	0
200	0	0		0		0		0		++++

* N designates salt solution shaken with activated charcoal (Norite).

† NaCl No. 1 plus the ash of charcoal that had been shaken with a toxic salt solution.

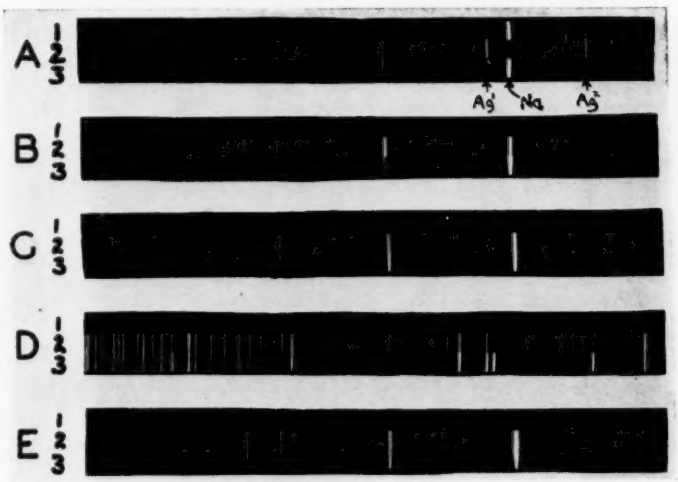
‡ NaCl No. 1 plus the ash of untreated charcoal.

that they caused no hemolysis of fish erythrocytes. A possible explanation for this seemed to be that the toxic principle had been adsorbed and removed by the fine particles that had been filtered off. Though this proved not to be the case, as will be shown later, it suggested attempting to remove the impurity from the toxic solutions by adsorption. Animal charcoal seemed to be the best adsorbent to use, since if the inorganic impurity was adsorbed it could be concentrated by subsequent ashing of the charcoal.

The results given in Table V show that when a toxic solution of sodium chloride is shaken with charcoal and the charcoal removed, the resulting solution has lost its toxic action. If this charcoal is now ashed, the ash treated with HCl, neutralized, and a small portion

added to a non-toxic solution of NaCl, hemolysis occurs. The ash of untreated charcoal also shows a faint hemolytic action though very unlike the pronounced action of the treated charcoal.

Since the adsorption of the impurity on charcoal furnishes a simple



EXPLANATION OF PLATE I

Ag^1—Silver line λ3280.67 A.U.
Ag^2—Silver line λ3382.89 A.U.
Na—Sodium line

A

1. Woods Hole Sea Salt (non-toxic)
2. $AgNO_3$
3. NaCl Brand No. 3 (toxic)

B

1. Graphite Electrodes
2. NaCl Brand No. 4 (non-toxic)
3. NaCl Brand No. 2 (toxic)

C

1. Graphite Electrodes
2. Table Salt No. 1 (non-toxic)
3. Table Salt No. 2 (non-toxic)

D

1. Graphite Electrodes
2. Ash of untreated charcoal
3. Ash of charcoal shaken with NaCl No. 2

E

1. Graphite
2. NaCl No. 3 made non-toxic by shaking with charcoal
3. NaCl No. 3 (toxic)

Obtained with Hilger E1 Quartz Spectrograph, using graphite arc and with technique similar to that described by Shipley, Scott, and Blumberg (1932).

means of purifying sodium chloride solutions for future use, it may be worthwhile to give in detail the procedure followed. To each liter of isotonic salt solution, two grams of animal charcoal (Norite) were added and shaken intermittently throughout the day. The solution

was then allowed to stand overnight and the charcoal filtered off in the morning, the filtrate then being ready for use. The removal of the impurity seems to be a slow process as the addition of more charcoal with more frequent shaking does not enable one to obtain a non-toxic solution in shorter time. It is possible that a small amount of sodium chloride is also removed by the charcoal, which would change slightly the concentration of the solution. This change would be significant only where extremely accurate work is contemplated. Purification of the salt by recrystallization is ineffective.

Since a concentration of the toxic material was now possible by adsorption on charcoal and subsequent ashing, a spectrographic identification of the inorganic impurity seemed possible. Attempts to duplicate the hemolytic action by addition of various inorganic salts to non-toxic solutions had failed.

The results obtained with the spectrograph are shown in Plate I [3] and indicate that silver is the impurity present in the sodium chloride that causes hemolysis of erythrocytes. Those brands of salt that showed hemolysis, Nos. 2 and 3, give the *raies ultimes* of silver. Woods Hole sea water and Brand No. 4, which do not cause hemolysis, contain no detectable silver. It was also impossible to detect silver in two brands of table salt that were non-hemolytic. It had previously been thought that isotonic solutions made from these salts were non-hemolytic due to the removal of the toxic principle by adsorption on insoluble matter contained in them which was filtered off before use.

A comparison of the spectrographs of the ash of charcoal that had been shaken with a toxic salt solution and of the ash of untreated charcoal show the silver lines to be much brighter in the former than in the latter. The presence of silver in the untreated ash explains the delayed hemolysis that resulted on the addition of this ash to a non-toxic salt solution (see Table V). Finally, a comparison of the spectrographs of a toxic sodium chloride before and after shaking with charcoal shows that the silver lines have completely disappeared due to the charcoal treatment. These results would seem to indicate that in whatever state silver exists in the sodium chloride solutions, it is readily adsorbed by activated charcoal, and that the presence of silver is always attended by hemolytic action.

It remained to be shown that hemolysis could be produced by the addition of small amounts of silver to a non-toxic salt solution. Since the marine fish that had been used in the first part of this investigation were not obtainable in Baltimore, it was necessary to use freshwater fish. Several specimens of trout and perch were obtained from the

[3] Run by Mr. Blumberg.

State Hatcheries.[4] The erythrocytes of both these species were hemolyzed rapidly both by a toxic salt solution and by a non-toxic salt solution plus a small amount of silver nitrate. A typical experiment is shown in Table VI.

It can be seen that when silver nitrate is added to a non-toxic salt solution to give a final concentration of added silver equal to 10^{-5} molar, hemolysis occurs in about the same time as in a toxic salt solution. This added silver can then be removed by shaking with charcoal and a non-hemolytic solution obtained. Solutions stronger in silver than 10^{-5} molar are not obtainable, as a precipitate of silver

TABLE VI

Hemolysis of Trout Erythrocytes by Silver

Solution number	1	2	3	4	5
	NaCl No. 2 0.15 M	Solution 1 shaken with charcoal and filtered	Solution 2 plus $AgNO_3$ to give $[Ag] = 10^{-5}$ M	Solution 2 plus $AgNO_3$ to give $[Ag] = 10^{-4}$ M	Solution 3 shaken with charcoal and filtered
Hemolysis Time *min.*					
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	S.G.	0	0
4	S.G.	0	+	0	0
5	++	0	++	0	0
6	+++	0	+++	0	0
7	++++	0	++++	0	0
8		0		0	0
9		0		0	0
10		0		S.G.	0
15		0		+	0
20		0		+++	0
30		0		++++	0

chloride results. Solutions made hemolytic by the addition of silver show the same reaction to pH as a toxic salt solution (see Table IV).

This leaves little doubt that silver is the toxic agent in sodium chloride that causes hemolysis of fish erythrocytes. From a comparison of the rates of hemolysis in a toxic salt solution and a non-toxic salt solution plus known amounts of silver, the concentration of silver in various brands of sodium chloride is found to be of the order of magnitude of 10^{-3} to 10^{-4} per cent by weight.

Other inorganic salts were tested for their hemolytic action. The following salts were added to a non-toxic salt solution to produce concentrations ranging from 10^{-3} to 10^{-6} molar: $AlCl_3$, $AuCl_3$, $BaCl_2$,

[4] Through the courtesy of Mr. Swepson Earle, Commissioner.

$BeCl_2$, $CaCl_2$, $CdCl_2$, $CeCl_4$, $CoCl_3$, $CrCl_3$, CsCl, $CuCl_2$, $FeCl_3$, $HgCl_2$, KCl, $LaCl_3$, LiCl, $MgCl_2$, $MnCl_2$, NH_4Cl, $NiCl_2$, $PbCl_2$, $PdCl_2$, H_2PtCl_6, RbCl, $RhCl_3$, $RuCl_3$, $SbCl_3$, $SnCl_2$, $SrCl_2$, $ThCl_4$, $TiCl_3$, TlCl, $UO_2(NO_3)_2$, VCl_2, $ZnCl_2$. Only mercury and palladium showed marked hemolytic action. Hemolysis by the mercuric salt was always accompanied by a turbidity of the solution. The palladium salt produced a rapid hemolysis giving a clear solution at dilutions similar to that for silver. That palladium is not the impurity present in sodium chloride can be easily shown by the addition of sodium iodide. Concentrations of palladium (10^{-5} to 10^{-6} molar) that cause hemolysis at rates similar to that of toxic salt solution show a pronounced brown coloration on the addition of NaI due to the formation of insoluble palladium iodide. The addition of iodides to a toxic salt solution causes no coloration but does delay hemolysis, probably because AgI is formed, which is more insoluble than AgCl.

The striking similarity in hemolytic action of silver and palladium is of interest for these two elements lie in entirely different groups. Since silver and mercury have been widely used as bactericidal agents if necessary, the possibility suggests itself that palladium might also be useful in this connection, especially since its chloride is so much more soluble than that of silver.

Discussion

In searching for a toxic inorganic impurity in sodium chloride, it is doubtful if silver would be among the first elements to be considered. The low solubility of silver chloride is a well-known fact, and according to the solubility product one would expect its solubility to be still lower in the presence of excess chloride ions. However, there is evidence in the literature to show that silver chloride tends to form soluble complexes in sodium chloride solutions. Pinkus and Berkolaïko (1930) have presented data to show that the solubility of AgCl increases as the concentration of sodium chloride rises. This fact, coupled with the knowledge that quantities of minute importance to the analyst are of extreme physiological significance, tend to lessen the incredibility of the toxic action of silver occurring as an impurity in sodium chloride.

As stated early in this paper, it was found that all brands of salt that showed hemolytic properties were mined. However, crude table salt which is also mined contains no silver, so that it seemed unlikely that the silver occurred in the natural deposits. After much inquiry it was found that the final step in the purification of crude mined salt for the chemical trade was carried out in silver-lined vessels because

less corrosion occurred in such vessels. This then would appear to be the source of the silver that occurs in some brands of sodium chloride.

The presence of silver in sodium chloride as observed here is not a chance occurrence. In a private communication, Dr. E. Wichers states that Mr. A. Isaacs found silver to be present when testing sodium chloride for suitability as a chemical reagent. He also points out that the specifications for sodium chloride published by the American Chemical Society (1928) indicate that silver may be found.

Since presenting this paper before the Society of Biological Chemists, there has come to my attention several instances in which the employment of an artificial biological medium composed of sodium chloride in whole or in part has proved unsuitable. If the brand of sodium chloride used was changed, usually to Kahlbaums, which has been found silver-free, the medium became satisfactory in all respects. That the toxic action of certain brands of sodium chloride is not limited to the hemolysis of fish erythrocytes has been shown by Williams and Jacobs (1931). These workers have found that salts which cause hemolysis usually also exhibit a more toxic action on various other marine organisms. It has also been observed in this laboratory that not only fish but also mammalian erythrocytes undergo hemolysis when placed in isotonic solutions made from sodium chloride containing silver. In the case of mammalian red cells the rate of hemolysis is in terms of hours instead of minutes, though the final effect is none the less definite. Controls showed no hemolysis after several days.

It would, therefore, seem highly appropriate to sound a note of caution in the indiscriminate use of sodium chloride in the preparation of physiological saline solutions, no matter what may be their intended use. Though the effect of the silver, occurring as an impurity, on other biological material may not be as obvious as in the case of fish erythrocytes, it may nevertheless be as serious. Unseen changes in the biological material may have occurred which will alter entirely the response to experimental procedure. The use of silver-free sodium chloride solutions in such work is therefore suggested. Such solutions can easily be prepared by the simple method of purification outlined above.

Summary

1. The hemolysis of fish erythrocytes by isotonic solutions made from certain brands of sodium chloride has been shown with the aid of the spectrograph to be due to silver occurring as an impurity in those salts.

2. The amount of silver present in such salts is of the order of magnitude of 10^{-3} to 10^{-4} per cent and originates from the use of silver-lined vessels in the purification process.

3. A simple procedure for the removal of silver from such salts by adsorption on charcoal has been described.

4. Palladium is the only other element that has been found to be as effective as silver in causing hemolysis.

5. Since the toxic action of silver is not limited to the hemolysis of fish erythrocytes, it is suggested that silver-free sodium chloride be used in the preparation of physiological saline solutions.

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THE RELATION OF THE THYROID AND THE HYPOPHYSIS TO THE MOLTING PROCESS IN THE LIZARD, HEMIDACTYLUS BROOKII

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The molting of the epidermis occurs with great regularity in many groups of lizards. In the Amphibia, where this periodicity is less regular, the thyroid has been shown to play an important rôle in the process (Adams, Richards, and Kuder, 1930). Complete cessation of molt has been reported in the common European lizard, *Lacerta*, following thyroidectomy (Drzewicki, 1928). Since the investigator experimented with only a small series of lizards and since we have observed that lizards in poor health are very irregular in their molting, it seemed highly desirable to repeat his work on a larger scale. We have selected for this purpose a lizard which molts at more frequent intervals than *Lacerta*. Environmental influences have been ruled out as far as possible by keeping both experimental and control series in a large incubator at a constant temperature and at a humidity which fluctuated only slightly. As already reported in a preliminary note (Noble and Bradley, 1931), we have obtained results very different from those Drzewicki reported. The present paper represents a detailed statement of our findings.

MATERIALS

The lizards we found most convenient to employ in this study were geckos of the species *Hemidactylus brookii*. Our entire series came from Samaná, Dominican Republic, where this species, imported from Africa, is now abundant. The lizards were maintained throughout our experiment in cages measuring 50 cm. long, 35 cm. deep, and 33 cm. wide, with glass sides, a removable iron bottom, and a screen top. A layer of sand, frequently changed, covered the floor of each cage, and the lizards were given ample hiding places among strips of cork bark introduced into the cages. From 15 to 20 lizards lived in each cage and since they rarely mutilated one another they appeared to have sufficient room. The lizards were fed with mealworms (*Tenebrio molitor*) and wax worms (*Galleria mellonella*) two, or rarely, three times a week. Fresh water was supplied daily. The series of cages

were arranged in rows in a large, glass-sided incubator maintained at a constant temperature of 28° C. and average relative humidity of 53.7 per cent. A fan was provided to distribute the heated air evenly throughout the incubator.

The lizards were numbered and individual records made from day to day. Molting is more rapid in the Gekkonidæ than in any other family of lizards. The head, body, and tail do not have independent molting cycles as in some iguanids. As the period of molt approaches the entire skin appears more opaque or gray. The superficial layers of epidermis are freed from the underlying layers over most of the lizard's body within a short time. Consequently, the lizard appears to be veiled over all its body with the white shed skin which always splits along certain lines of the body. The lizards rub off some of the skin but most of it they pull off with their jaws, usually swallowing it piece by piece. The lizards aid one another in this dispensing with the old skin. The habit is annoying to the observer because it may result in a complete removal of the skin within a few hours. Usually the molted skin is entirely shed within 24 hours after it first loosens from the body. The opaqueness of the skin to be shed is discernible one or two days before actual loosening occurs. A recently shed lizard has a bright integument very different from the opaque skin of the day preceding molt. Although in most cases some pieces of skin were found as evidence of a molt, in a few cases we had to rely on the change in skin opacity reported in our daily record as proof that a molt had occurred.

The Molting Cycle

From September 1930 to December 1931, we made molting records on 123 individuals of *Hemidactylus brookii*. We have 247 records of molt in this series of lizards which represents the control series of the several experiments to be detailed below. These 123 lizards molted on the average every 15.73 days. Our minimum period between molts was 11 days, our maximum apparently 23.2, these extremes being the normal rate for a very few healthy individuals, and almost all other records falling near the average. *H. brookii* is a tropical species and when maintained in good health at a constant temperature it shows a remarkable regularity in its molting periodicity.

Effect of Thyroidectomy on Molting

In January 1931, we selected a series of the lizards and removed their thyroids under the binocular microscope. It was found advisable to make an incision across the whole width of the throat immediately anterior to the clavicles. The muscles overlying the thyroid

were either cut or spread apart. The several lobes of the thyroid were then gently freed of their attachment by means of a fine forceps and the blood vessels pinched off with the same instrument. In spite of the extent of the incision little bleeding occurred and this was never sufficient to cause death. After removing the thyroid, the muscles were drawn together with three or four separate stitches of fine silk thread, and the skin snapped together with skin clips. An antiseptic adhesive powder was shaken on the wound. By this time the lizards had usually recovered from the ether. Controls were opened in exactly the same way and the thyroid pulled away from the surrounding tissue sufficiently to cause some bleeding. Thyroidectomy apparently did not impair the health of the lizards. Immediately upon recovering from the effects of etherization they were as active as before, and they continued to behave as normal *Hemidactylus*. The death rate among these animals was no higher than that among normal ones.

Most of the lizards in the series were allowed to live until they died but many were killed after they had shed a sufficient number of times following the operation. The throats of the experimental lizards were sectioned serially and examined carefully for the presence of thyroid follicles. Some lizards died too soon to show any deviation in their molting. The others, numbering 79 in all, will be considered here. Of these 63 were thyroidectomized, the other 16 being incised controls. Upon comparing the results of the histological examinations with the post-operative molt periods, it was found that a thyroidectomized lizard having 7 or more follicles left in its throat shed at exactly the same intervals as the controls; but that one having 6 or less follicles left shed like completely thyroidectomized animals. Of the 63 thyroidectomized, 15 were found to have 7 or more follicles left, and 13 were found to have 6 or less follicles left. The former group we will call incompletely thyroidectomized; the latter we may group in the following account with the 35 completely thyroidectomized ones. Two apparently completely thyroidectomized lizards had molting periods like controls. In averaging these with the completely thyroidectomized group we tend to destroy the sharp difference which exists between the molting periods of the two groups. One lizard which had more than 7 follicles shed like a completely thyroidectomized lizard but was averaged with the incomplete group, and tended to break down the distinction further.

The average of the completely thyroidectomized group was 26.47 days (variation 16.33–49.0), while that of the controls was 16.43 (variation 14–20.25). The average of the incomplete group was 18.55 days (variation 14.57–21). Thyroidectomy, then, in spite of the three excep-

tions noted above lengthens the average molt period of *H. brookii*. In this species it does not, however, inhibit molt entirely.

Removing the thyroid of these lizards did not delay markedly the appearance of the molt immediately following the operation. In the group of 48 completely thyroidectomized lizards (including the 13 with 6 or less follicles) this molt appeared, on the average, 17.72 days after the preceding one. In the incompletely thyroidectomized series the average time was 16.3 days while in the controls it was 15.12 days. There is, therefore, a distinct lag in the effect of thyroidectomy on the molting of the lizards. Since the lag was approximately the same whether the operation was performed in the early or later part of the interval between molts, it would seem that at any one molt the tissues become so adjusted that the following molt will occur at approximately the usual time regardless of any interference with the process.

Effect of Thyroxin

To establish that the delay in molt of thyroidectomized lizards was due to the absence of the thyroid and not to some secondary effect of the operation, it seemed desirable to show that the molting periodicity could be returned to normal in thyroidectomized lizards by replacement therapy. Unfortunately our series of geckos was not sufficiently large to sacrifice a number for their thyroids. We have therefore used thyroxin as a substitute. Hopping (1931) has showed that 3 days after injecting an alligator with thyroxin the tissue metabolism is raised 150 to 190 per cent above the control. Hopping used Squibbs thyroxin and we have obtained our thyroxin from the same source.

Eight lizards were selected for thyroxin treatment. Four were completely thyroidectomized, as later histological examination showed, and they were shedding at long intervals. Two were incised controls of the thyroidectomy series and 2 were normal lizards which had not been opened in the manner of the others. They received 0.3 cc. of a 1 to 10,000 solution of thyroxin on alternate days. The injections were given intramuscularly in the hind legs. After two weeks the thyroxin solution was weakened to 1 to 30,000, but the same amount was given at the same intervals.

Five other lizards were selected for the control series. Two were thyroidectomized lizards shedding in long periods. Subsequent histological check revealed that one of these controls had 2 and the other 5 follicles, but as stated above this was not sufficient thyroid tissue to keep the molting periodicity up to normal. Two other controls were stock lizards and one was an incised control. The five lizards received 0.3 cc. of salt solution (.6 per cent) at the same intervals as the experimentals.

None of the lizards stood the injecting well. In none, no matter how early in the inter-molt period the injecting was begun, was the time of appearance of the first molt following the initial injections altered materially. The next molt, however, was distinctly influenced by the thyroxin. In one thyroidectomized lizard whose molts had averaged 28 days apart, the second molt following the thyroxin treatment came 17 days after the first. Another thyroidectomized lizard with a cycle of 25.8 days shed 19 days after the first molt and then 18 days later. A third thyroidectomized lizard with a cycle of 28.2 days between molts shed in 16 and then in 18 days for the same two periods. Unfortunately, these three lizards did not live to give additional records, but it will be noted that the records obtained represent a return to the normal molting periodicity of the species. The thyroxin did not shorten the intervals between molts in the lizards having their thyroids intact. One of the incised lizards had two molts at the usual intervals and the other three molts at the same average time following the first molt after treatment. The two lizards which had not been incised died before giving any record, and the same was true of one of the thyroidectomized lizards.

The control series receiving the saline solution did not shorten the intervals between their molts. A thyroidectomized individual which has been molting on the average every 29.8 days, lengthened the period following the first molt after treatment to 32 days. An incised control shed at the usual time for the second molt. The other three controls did not live to the time for their second molt. It would seem from this record that 0.3 cc. of salt solution was too great an amount for the health of the lizards. However, the solution did not dilate the limbs greatly and we are inclined to believe that the lizards died from infections received during their frequent handling.

It seemed to us strange that thyroxin would return the delayed molt of thyroidectomized lizards to the normal and yet would not shorten the interval between molts in the unoperated controls. We therefore repeated the thyroxin experiments on other unoperated lizards but secured only similar results. CH 345 was injected 13 times with 0.25 cc. of a 1 to 3,000 solution of thyroxin over a period of 19 days. During this period it molted twice, the first time 12 days after beginning the treatment and the second time 13 days later. CH 67 received 24 injections of thyroxin, 0.25 cc. each time. Five of these injections were 1 to 10,000, 2 were 1 to 6,000, and 17 were 1 to 3,000. These injections were extended over a 34-day period. During this time the molts came at the following intervals: first at 15 days after the previous molt, then at 13 days, and finally at 15 days.

We have also attempted to change the interval between molts by implanting fresh thyroids of other species of lizards, by injecting various solutions of desiccated thyroid (Lilly) and by various solutions of adrenalin chloride. None of these treatments gave clear-cut results. In none was the average periodicity of molt distinctly increased over that of the controls.

Effect of Hypophysectomy

Removal of the anterior pituitary in Amphibia and some other vertebrates is known to inhibit the normal functioning of the thyroid. If extirpation of the thyroid delays the molting process in lizards, hypophysectomy should have a similar although less marked effect. We have, therefore, hypophysectomized a series of *H. brookii* and compared their molting records with the thyroidectomized series. The hypophysis was removed through the roof of the mouth, care being taken to avoid injury to the base of the brain. In each case we attempted to remove the entire hypophysis but were not always successful.

Twenty-six hypophysectomized lizards survived the operation and gave us 60 molting records. In this series the interval between molts dropped from a 15.73-day average to a 23.21-day average, with a range of from 14 to 39 days. Hypophysectomy, therefore, definitely lengthens the period between molts.

The heads of 10 of these 26 hypophysectomized lizards were sectioned and examined for remnants of the pituitary. It was found that one specimen, No. 57, which had the pars posterior intact, part of the pars intermedia left, and also some of the pars anterior, did not markedly lengthen its post-operative molt periods, which were 16.5, 16, 20, and 19 days. Two other specimens, Nos. 160 and 168, had only one molt period before they died and in each case it was a short one, 14 and 19 days respectively. As will be shown in other specimens, this does not necessarily mean that their subsequent periods would have been short if they had lived. Of these two, No. 160 had only a small portion of the pars posterior remaining, and No. 168 had no hypophysial tissue left. The other 7 specimens all had long periods and showed no remnant of the pars anterior.

Five of the 7 having no pars anterior left had part of the pars intermedia, part of the pars posterior left, or both. No. 124, whose post-operative periods were 19, 25, 26, and 27 days, had the pars posterior intact and portions of the intermedia left. No. 137 had some intermedia left, but no posterior, and shed in 18 and then 36 days. No. 150 had the whole posterior and intermedia left intact and shed

in 25, 41, and 43 days. No. 130 had only a portion of pars posterior left but no intermedia and shed in 22, then 26 days. No. 235 had almost the whole posterior intact but no intermedia and shed in 19, 25, 31, 35, 39, 30, 29, 39, and 28 days, respectively. From the above record it is clear that either the whole or part of either the pars intermedia or pars posterior or both together are not sufficient to maintain molt periods of normal length. Pieces of the pars anterior lobe are necessary to accomplish this.

Two of the 10 hypophysectomies sectioned had no hypophysial tissue left. Number 58 shed in 14, 21, 34, 23, and 31 days. Number 134 shed in 23 days. These periods produced by lizards with no hypophysial tissue left are no longer than those produced by lizards with any amount or combinations of intermedia and posterior left. The only specimens keeping their periods within the limits of normal unoperated lizards were those having some anterior left. The pars anterior, then, influences the length of molt periods and not the pars posterior nor the pars intermedia.

The hypophysis also controls the expansion of the melanophores. It is usual for these lizards to remain quite dark under the conditions of temperature and lighting imposed by these experiments. Of the two animals, Nos. 58 and 134, which had no hypophysial tissue left, one remained very light colored from the time of operation until death. All other specimens having no pars intermedia left, *i.e.* Nos. 130, 160, 168, and 235, but which did have either some pars anterior or pars posterior left were reported as being continuously light colored. Two specimens, No. 137 and No. 57, which have some pieces of intermedia left are reported as being darker. Another, No. 124, which had some intermedia pieces, was still darker. The lizard, No. 150, which had its entire intermedia present was always dark; *i.e.* normal color.

The hypophysectomized lizards exhibited some variation in the length of time required to complete a molt once it had begun. We could not secure definite evidence that the variation was correlated with the degree of completeness of the operation. For example, one lizard in our series, No. 58, was proved by histological examination to be completely hypophysectomized, all three lobes having been removed, and yet it shed as rapidly as the controls. Another lizard, No. 168, was also completely hypophysectomized but remnants of the old shed remained attached to the skin for long periods. It is possible that a serious delay in completing the molt is correlated with some injury to the brain suffered at the time of hypophysectomy. Our sections, however, gave no evidence of such an injury.

Discussion

Thyroidectomy greatly lengthens the period between molts in the gekkonid lizard, *Hemidactylus brookii*. It does not delay the rate at which the molt layer is freed from the underlying tissues and removed by the lizard. In this it stands in contrast to hypophysectomy, which both increases the period between molts and frequently, but not always, delays the process of loosening and freeing the epidermal layer to be cast. Since northern reptiles do not molt in winter, it is apparent that molting is closely correlated with the metabolic rate of reptiles. The thyroid is well known to have a marked influence on the metabolic rate of many vertebrates. The question arises whether the influence of thyroidectomy on molt is indirect, or whether thyroid function is closely bound with the molting process, as has been claimed in Amphibia.

In Amphibia at the time of metamorphosis, when it has been shown that the metabolic level is greatly increased, molting occurs for the first time in the life of the individual on a large scale. In the newt, Springer (1909) found that well-fed newts molted more frequently than less-fed controls. How greatly the metabolism of these newts was increased is not known. On the other hand, all molting in Amphibia does not appear to be correlated with increased metabolic rate. Ružička (1917) obtained an increase in the number of molts in European newts by starvation. Adolph and Collins (1925) found that gauze bandage would increase the frequency of molt in the common newt. Dawson (1920) found a molt followed drying the skin in *Necturus*. As long ago as 1894 Nicolas found that operating on a salamander induced a molt. Injury of the integument has been shown by Adolph and Collins, as well as by other workers, to call forth a molt. Obviously, excitation of the integument of Amphibia by various methods speeds up the appearance of the next molt. In Amphibia there appears to be a dual control over the mechanism of molt, one apparently hormonal and the other nervous. The endocrine and nervous systems may function vicariously in the case of the contraction of melanophores, and of smooth muscle cells. Possibly thyroid and nervous impulses to the integument both cause the molting cells to release their secretion, causing the superficial layer of the epidermis to be lifted from subjacent tissues. However, Adams and Grierson (1932) found that pilocarpine did not increase the incidence of molt in the newt, although this drug has been shown to cause release of secretion from the unicellular hatching glands of salamanders (Noble and Brady, 1930).

In reptiles the molting cells reported in Amphibia do not appear

and molting is far more regular in its periodicity. In many snakes and some lizards a fluid has been found under the molted skin at the moment of shedding. This fluid has the appearance of lymph. It is possible that a similar fluid is extruded during the molting process of all reptiles, but in most lizards it dries before the old skin is actually shed. Adams and Grierson (*op. cit.*) report a fluid under the molting skin of newts which molted out of the water. They infer that this fluid is lymph and not the secretion of the unicellular hatching glands which have been described in other Amphibia. From the data at hand it is impossible to state how great a rôle the extrusion of lymph through the skin plays in the molting of either reptiles or Amphibia. Since, however, the molting of reptiles is not hastened by the various excitations reported in Amphibia, the mechanism of molt is apparently different in the two cases.

Thyroidectomy and hypophysectomy may delay the molt of reptiles merely by lowering the rate of metabolism. Molting is delayed by low temperatures and here the metabolism is obviously affected. Since molting occurs in *Hemidactylus* in the total absence of the thyroid, this gland is not an essential part of the molt mechanism.

Conclusion

Either thyroidectomy or hypophysectomy lengthens the period between molts in the lizard, *Hemidactylus brookii*, but neither operation entirely prevents molting. Thyroidectomy does not delay the appearance of the molt immediately following the operation but affects all later molts. If 6 or fewer follicles remain after thyroidectomy, the effect is the same as complete thyroidectomy. Injections of thyroxin, 1 to 10,000 and later 1 to 30,000 given intramuscularly to thyroidectomized lizards on alternate days, bring a return of the molt to its normal periodicity. However, neither injections of thyroxin nor implantations of fresh lizard thyroid increase the frequency of molt in the intact lizard.

Completely hypophysectomized *Hemidactylus brookii* turn a pale grey and remain this color permanently under varying environmental conditions. Lizards with fragments of the pars intermedia remaining turn a slightly dark tone under conditions where the controls are very dark grey.

Removal of the pars anterior of the pituitary alone has the same effect on delaying the molt as removal of the whole gland. The lengthening of the interval between molts may be due to a lowering of the metabolic rate of the lizard and not to any direct action of either the anterior pituitary or the thyroid on the molt mechanism.

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THE EFFECT OF ADRENALIN CHLORIDE AND TOAD VENOM ON THE BLOOD PRESSURE AND HEART RATE OF THE TROPICAL TOAD, BUFO MARINUS

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The tropical toad, *Bufo marinus*, secretes and stores in its skin glands a large amount of venom containing powerful adrenalin and digitalis-like substances without apparent harm to itself. Various workers have determined the pharmacological action and the minimum lethal dose of toad venom for animals other than the toad (Vulpian, 1854; Phisalix and Bertrand, 1893; Abel and Macht, 1912; Chen, Jensen, and Chen, 1931; Xavier, Vellard, and Vianna, 1931). Vulpian (1858) stated that the venom of the toad, which destroyed the irritability of the heart of the frog and of the salamander, had no action on the heart of the toad. Kobert (1887) found the vessels of the toad when perfused with toad venom to be less affected than those of the frog. Abel and Macht (1912) showed that both bufo-epinephrin and "bufagin," isolated from the venom, caused vasoconstriction when added to Locke solutions used to perfuse the blood vessels of *Bufo marinus*, and stated that the toad was relatively immune to "bufagin" but not to epinephrin. In the South African clawed toad, *Xenopus lævis*, Gunn (1930) found an adrenalin-like substance in the skin secretion which produced striking circulatory effects in the cat and the rabbit, but neither the skin secretion nor adrenalin had an effect on the circulatory system of *X. lævis*.

No reference in the literature could be found concerning the effect of adrenalin chloride or of toad venom on the blood pressure of the toad, nor even in any way concerning the measurement of blood pressure in this amphibian. Bieter and Scott (1929) found that an intravenous injection of 0.2 cc. of adrenalin chloride, 1 : 10,000, gave a rise of blood pressure lasting one hour and a quarter in the frog, *Rana pipiens*. The present report concerns the effect of intravenous injections of adrenalin chloride and of toad venom on blood pressure and heart rate in *B. marinus*. A determination of the minimum amounts of these substances necessary to produce a rise in blood pressure on intravenous injection is also reported.

Method

Specimens of *B. marinus* weighing from 215 grams to 590 grams were prepared by destroying the fore-brain through the *os fronto-parietale* and pithing the spinal cord posterior to the second vertebra. The blood pressure was recorded by a mercury manometer of 3.5 mm. bore from a cannula in the *A. femoralis*. Sodium citrate, 7 per cent, was used as an anticoagulant and the cannula was wet with heparin before insertion. Injections were made with a Luer tuberculin syringe through a cannula in the *V. femoralis*. For threshold determinations the volume of fluid was generally not more than 0.2 cc. An intravenous injection of 0.5 cc. of amphibian Ringer's solution was without effect. Adrenalin chloride (Parke, Davis and Co.) was used in dilutions with Ringer's solution from 1 : 50,000 to 1 : 50,000,000. The venom was expressed on a weighed cover-glass from the large gland behind the ear, weighed, dissolved in Ringer's solution to known dilutions, and injected immediately. Laboratory temperatures varied from day to day between 27° C. and 29° C.

Results

The average systolic blood pressure in the femoral artery in nineteen toads about one-half hour after the destruction of the cerebral hemispheres and spinal cord and before injection was 33 mm. Hg, ranging from 22 mm. Hg to 56 mm. Hg. The heart rate was 76 per minute and the respiration 106 per minute. The pulse pressure varied from 4 mm. Hg to 12 mm. Hg. In two toads under ether anesthesia, with the nervous system intact, the blood pressure was 32 mm. Hg, the heart rate 64, and the respiration 106.

In twenty-four toads an intravenous injection of 0.2 cc. of adrenalin chloride 1 : 50,000, which was 0.8 μg. to 2 μg. per 100 grams (1 μg. equals 0.001 mg.), produced a rise in systolic blood pressure of 21 per cent to 155 per cent which lasted from 2 to 6 minutes (Fig. 1, *A*). Weaker doses such as 0.2 cc. of 1 : 200,000 regularly produced a pressor response; thus in one instance this dose was 0.33 μg. per 100 grams and gave a rise of 56 per cent. Subsequent doses were just as effective as first doses. The minimal effective dose of adrenalin chloride for a pressor response was found in nine toads to average 0.05 μg. per 100 grams with a range from 0.02 μg. to 0.09 μg. No depressor responses occurred even with doses as low as 0.002 μg.

While the effect of adrenalin on the heart rate in these preparations was not uniform but varied with the strength of dose and condition of the animal, there was generally a decrease. In fifteen toads doses from 0.2 cc. of 1 : 200,000 to 0.5 cc. of 1 : 50,000 (0.33 μg. to

2.5 μg. per 100 grams) gave a fall in heart rate of 6 per cent to 67 per cent associated with the rise of blood pressure. In three cases a rise of heart rate of less than 10 per cent occurred with a relatively large dose (1 μg. per 100 grams). In one case with a low heart rate of 44 per minute a dose of 2 μg. caused an increase to 56 per minute, while twenty minutes later a dose of 2.5 μg. caused a fall in heart rate from 68 to 57.

An intravenous injection of toad venom in Ringer's solution produced a rise in systolic pressure in ten toads in all doses from 160 μg.

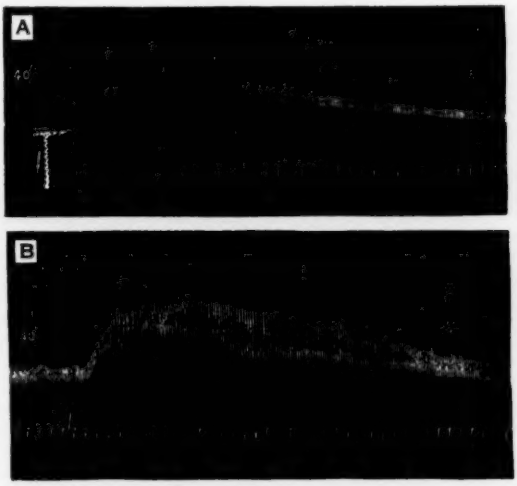

FIG. 1. *Bufo marinus*, 450 gram ♀, 29° C. Effect of intravenous injections on blood pressure and heart rate. *A*. Adrenalin chloride, 0.9 μg. per 100 grams. Rise in blood pressure, 145 per cent. Fall in heart rate, 9 per cent. Minimal pressor dose, 0.04 μg. *B*. Toad venom, 71 μg. per 100 grams. Rise in blood pressure, 123 per cent. Fall in heart rate, 22 per cent. Minimal pressor dose, 0.7 μg. Time in five-second intervals.

to 4 μg. somewhat in proportion to the amount of venom (Fig. 1, *B*). Thus, for example, 160 μg. gave a 292 per cent rise, while 15 μg. and 4 μg. gave 83 per cent and 33 per cent respectively. The minimum effective dose for a pressor response was found in five toads to average 0.5 μg. per 100 grams, with a range from 0.32 μg. to 0.71 μg. No depressor responses occurred with the small doses.

In nine toads the intravenous injection of the diluted toad venom caused a decrease (maximum 40 per cent) in the heart rate associated with the rise in pressure. In one case an increase occurred both with the venom and with adrenalin.

Discussion

The cardio-inhibitory effect of adrenalin associated with a rise of blood pressure suggests the presence of a reflex mechanism similar to that in mammals and certain lower vertebrates since in both the elasmobranch, *Squalus acanthias*, and the amphibian, *Necturus maculosus*, Lutz and Wyman (1932*a*, 1932*b*) have shown a reflex cardio-inhibitory mechanism stimulated by increased intravascular pressure.

In the reflex toad preparation the cardio-inhibition following the injection of venom must be due partly to the direct effect of "bufagin" (Abel and Macht, 1912) or cinobufotenine (Chen, Jensen, and Chen, 1931), although there was nothing in the response either of the blood pressure or of the heart to distinguish the effect of the venom from that of adrenalin alone.

Dragstedt, Wightman, and Huffman (1928) found the minimal effective pressor dose of adrenalin in the unanæsthetized dog to be less than from 0.2 cc. to 0.4 cc. of 1 to 1 million per kilogram per minute (0.02 μg. to 0.04 μg. per 100 grams). In rats, under urethane, Wyman and tum Suden (1932) generally obtained a detectable pressor response to adrenalin with 0.02 μg. per 100 grams, but in normal rats they found that 0.04 μg. per 100 grams was necessary to produce a visible vasoconstriction of the mesenteric vessels. These figures when compared with the pressor threshold for *B. marinus* to adrenalin (0.02 μg. to 0.09 μg. per 100 grams) indicate that this amphibian and the mammal are about equally sensitive to adrenalin. The total amount present at any time in the skin glands of the toad, the secretion of which yields about 5 per cent adrenalin according to Abel and Macht (1912), must be a great many times that present in the adrenal of the mammal (Sollman, 1932). According to Chen, Chen, and Jensen (1932) the adrenalin of the skin glands does not enter the blood stream although Phisalix and Bertrand (1893), using a questionable procedure, believed they found the toad venom in the blood in sufficient quantity for biological assay.

Since no depressor responses were obtained with adrenalin in doses as low as 0.002 μg. (one twenty-fifth the average pressor threshold), it appears that there are no vasodilators present in *B. marinus* sensitive to adrenalin.

Summary

1. Adrenalin chloride injected intravenously in *Bufo marinus* in doses of 0.33 μg. to 2 μg. per 100 grams produced a rise in systolic blood pressure of 21 per cent to 136 per cent. The average minimal effective dose was 0.05 μg. per 100 grams, ranging from 0.02 μg. to 0.09 μg.

2. Doses of 0.33 μg. to 2.5 μg. per 100 grams generally caused a decrease in heart rate of 6 per cent to 67 per cent associated with the rise of blood pressure.

3. Toad venom injected intravenously in *Bufo marinus* in doses of 4 μg. to 160 μg. per 100 grams produced a rise of systolic blood pressure of 33 per cent to 292 per cent. The average minimal effective dose was 0.5 μg. per 100 grams, ranging from 0.32 μg. to 0.71 μg.

4. The vasoconstrictor mechanism of *Bufo marinus* is one-tenth as sensitive to its venom as to adrenalin.

5. The response to adrenalin in *Bufo marinus* suggests the existence of a reflex cardio-inhibitory mechanism stimulated by increased intravascular pressure.

6. No vasodilators are present in *Bufo marinus* sensitive to adrenalin chloride in doses down to one twenty-fifth of the minimal effective pressor dose.

7. The vasoconstrictor mechanisms of *Bufo marinus*, of the dog, and of the rat are about equally sensitive to adrenalin.

I wish to thank Dr. J. F. G. Wheeler, Director of the Bermuda Biological Station for Research, for placing the facilities of the station at my disposal.

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THE STRUCTURE OF EPIDERMAL MELANOPHORES IN FROG TADPOLES

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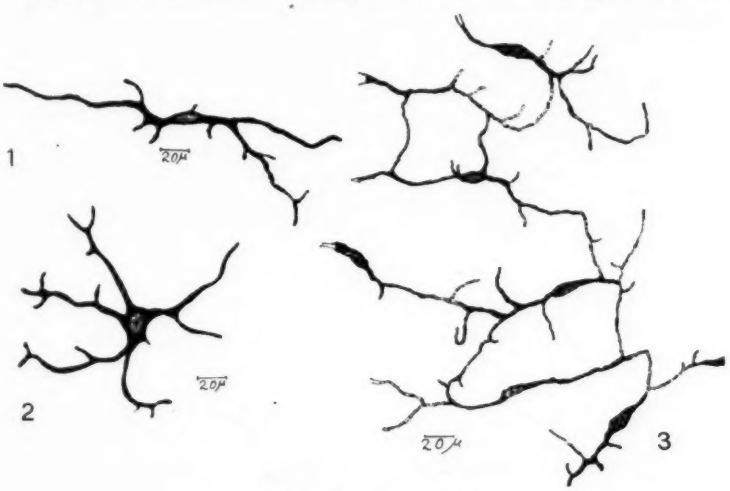
Color changes in animals have been the object of much investigation. Parker (1930), in an historical account of the subject, indicates that color changes have been recognized from very early times and are still a fertile field for study. Many problems concerning the reactions of pigment cells have been worked out. The structure of the pigmentary elements, however, has been difficult to determine. The main currents of opinion concerning the structure and the mechanism of "expansion" and "contraction" of the melanophores may be ascertained from recent papers by Hewer (1923), Matthews (1931), and Perkins and Snook (1932).

Hewer states that no fewer than one hundred and fifty papers have been published on color changes of amphibians alone. Even with this extensive work on amphibians little mention is made of the *epidermal* melanophores of frogs and their tadpoles.

In the frogs and tadpoles that I have studied, *Rana clamitans*, *R. catesbeiana*, *R. sylvatica*, *R. pipiens*, and one species of *Hyla*, two types of melanophores are present in the skin. Those of the dermal type as described by Hewer are very similar to the dermal melanophores of other vertebrates. In contrast to the dermal type, the melanophores found in the epidermis are greatly elongated and usually possess few branches (Figs. 1, 2, and 5). The usual length of single epidermal melanophores is from 150 to 200 μ. One cell 246 μ long was found but this may not be the greatest length to which they occur. It is at times difficult to ascertain the exact length of certain melanophores because of anastomosing of branches. Anastomosing of branches is rather common in regions where the melanophores are close together (Fig. 3). In about the middle of each epidermal melanophore there is a widened region which the writer has called the "cell body." The cell body is usually 15 to 18 μ in length and 7 to 10 μ wide. The branches average about 3 μ in width. The ellipsoidal nucleus lies within the cell body. The melanophores are not located at any particular level of the epidermis but they have never been observed in the cuticular layer of cells (Fig. 6). One part of a melano-

phore may be at a different level in the epidermis from that in which another part lies.

Observations on fully expanded melanophores in preserved or freshly excised skin are fairly easily made and the outlines of the cells may be studied in detail. This method of study, however, does not reveal, necessarily, the exact structure of the melanophore. A melanophore as seen in the "contracted" phase contains all or nearly all of the pigment within the cell body (Figs. 4 and 7), giving it an appearance strikingly different from that in the "expanded" phase in which the pigment is distributed throughout the branches (Figs. 1 and 5).



EXPLANATION OF PLATE 1

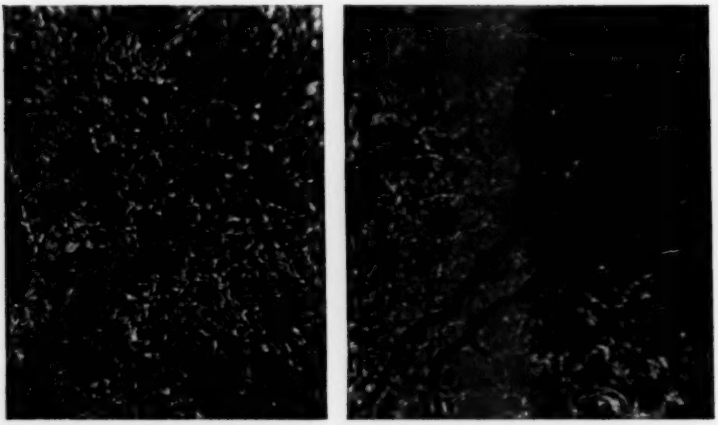
FIGS. 1 and 2. Camera lucida drawing of expanded epidermal melanophore of frog tadpole. From living, uninjured tissue.

FIG. 3. Camera lucida drawing of partly expanded epidermal melanophores showing anatomosis of processes. From living, uninjured tissue.

Immediately the question is raised as to the nature of the change from one phase to the other.

Hewer (1923) and Matthews (1931) have described melanophore change as a migration of the pigment granules within permanent processes. Their conclusions were that the processes did not change in size or shape, the granules of pigment migrating through the semi-fluid protoplasm of the processes. These conclusions were drawn from studies on the dermal melanophores of the frog and those of *Fundulus*. There is considerable evidence that the same condition exists in the epidermal melanophores of tadpoles.

In order to study melanophores in undamaged tissue, the method used was that described by Herrick (1932) in studying migrating epidermal tissue. By this method magnifications up to nearly 1000



EXPLANATION OF PLATE II

FIG. 4. Photomicrograph of contracted epidermal melanophores of frog tadpole. Living, uninjured tissue.

FIG. 5. Photomicrograph of expanded epidermal melanophores of frog tadpole. Living, uninjured tissue.

diameters were used successfully with the animal under only light anesthesia. Observations in some instances were made on a single melanophore over a period of several hours. The difficulty investi-

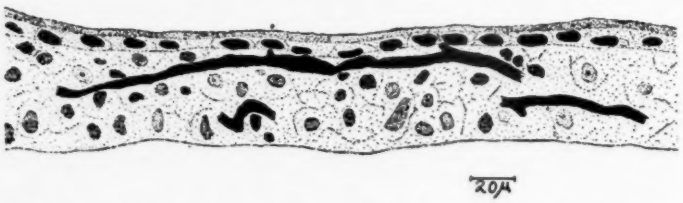
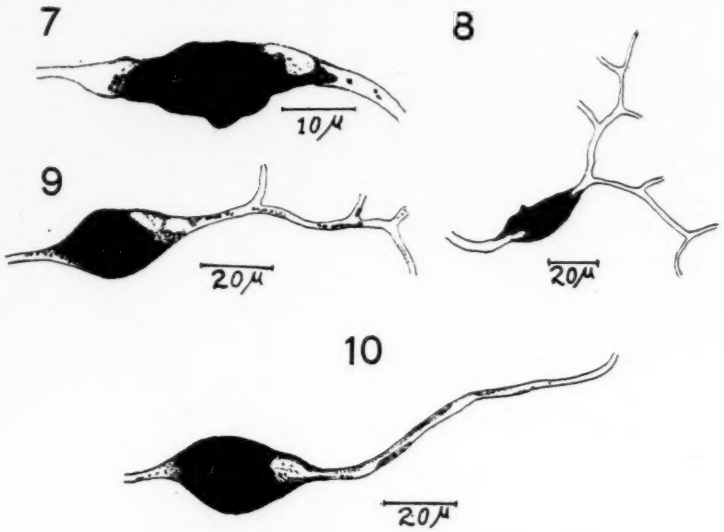


FIG. 6. Camera lucida drawing of section of tadpole epidermis showing positions of epidermal melanophores. Only fragments of melanophores are seen.

gators have encountered has been to observe in living tissue the processes of the melanophores when devoid of pigment. The walls of the processes are very thin and have about the same refraction as the surrounding tissue. With the aid of the apparatus I used, however,

it was possible to distinguish clearly the processes of the epidermal melanophores when devoid (contracted phase) of their pigment (Figs. 8, 9, and 10). This observation proves that the processes are not temporary and that contraction is not dependent upon their reduction in size. Hewer considered it very significant evidence, in support of his conclusion that the processes were permanent, when he found scattered pigment granules some distance from the cell body during



EXPLANATION OF PLATE III

FIG. 7. Camera lucida drawing of cell body of contracted epidermal melanophore showing nearly all of the pigment contained within the cell body. From living, uninjured tissue.

FIG. 8. Camera lucida drawing of contracted epidermal melanophore of frog tadpole showing processes devoid of pigment. From living, uninjured tissue.

FIGS. 9 and 10. Camera lucida drawing of nearly contracted epidermal melanophore of frog tadpole showing scattered pigment granules in the processes. From living, uninjured tissue.

the contracted phase. This condition was found repeatedly in the tadpole (Figs. 9 and 10). These single granules moved about slightly as is usually the case with pigment granules wherever they are found.

Expansion of the melanophore is a process of dispersion of the pigment granules which had been congregated within the cell body. The length of time required for melanophores to expand maximally depends upon the nature and the intensity of the stimulus. The epi-

dermal melanophores of large tadpoles, after intraperitoneal injection of 0.2 cc. of obstetrical pituitrin, expanded maximally in about 30 minutes. Animals placed in small aquaria, the sides and bottom of which had been covered with dead-black paper, became adapted to the dark background (almost maximal expansion of the melanophores) in from one to three hours or even longer. The individual pigment granules move so slowly that motion is barely perceptible under the microscope. There was never any suggestion of Brownian movement, a finding which is in contrast with the observations of Perkins and Snook (1932) in crustaceans. The rate of movement of a single granule was not uniform over a period of minutes and often differed from that of other granules in the same general area. The pigment most remote from the cell body was not massed together; instead, the granules were much scattered, in no wise suggesting a pseudopodium-like process being thrust out.

Contraction was a reversal of the process of expansion. During contraction the distal pigment was considerably scattered, with individual granules trailing far behind the main mass. With the employment of an ocular micrometer, no difference in the diameter of the cell body could be ascertained in the expanded and contracted phases.

Conclusions

1. Anastomosing of processes of epidermal melanophores in frog tadpoles is common.

2. The usual length of epidermal melanophores is from 150 to 200 μ.

3. "Expansion" and "contraction" are accomplished by the migration of pigment granules into and out of permanent processes.

4. Melanophore processes devoid of pigment were observed in living, undamaged tissue.

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THE INTRACELLULAR CANALICULI OF THE PHARYNGEAL GLANDS OF THE HONEYBEE [1]

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Recent studies of the intracellular secretory canaliculi in the salivary glands of the grasshopper and in the parietal cells of the stomach (Beams and King, 1932*a* and *b*) have reëmphasized the presence of these remarkable structures in certain types of gland cells. It is indeed surprising that they have received so little attention in current cytological literature unless perhaps due to an inherent skepticism derived from Holmgren's mistaken conception of them. The evidence seems decisive that these structures, together with the secretory material in the salivary glands of *Chironomus* larvæ (Krjukowa, 1929; Beams and Goldsmith, 1930; and Beams and King, unpublished), have been erroneously interpreted by Parat and Painlevé as Golgi apparatus or "vacuome." This unfortunate oversight on the part of Parat and his associate has led them to introduce on an unsound basis a new conception regarding the significance of these peculiar structures.

The pharyngeal glands of the honeybee offer exceptionally favorable material, not only for the study of intracellular secretory canaliculi, Golgi bodies, mitochondria, and the formation of secretion globules, but also for a study of the topographical relationship between these cytoplasmic components and secretory inclusions. Accordingly, this study was undertaken in an effort to advance the analysis of the question and significance of the intracellular canaliculi, Golgi bodies, mitochondria, and their relationship, if any, to the formation and the elimination of the secretory material by the cell.

MATERIAL AND METHODS

The material used in this study consisted of the pharyngeal glands of the worker honeybee. The head of the bee was placed on a glass slide in a drop of normal saline under the dissecting microscope and a median incision was made, dividing the head into two parts. By use of dissecting needles the glands were removed from the anterior lateral region of

[1] Part of this work was done at the Marine Biological Laboratory during the summer of 1932.

the head cavity and if whole mounts of the entire gland were desired, which incidentally is the best and simplest way to demonstrate the intracellular canaliculi, the remains of the head were removed from the area surrounding the glands and a cover glass applied. This whole operation takes less than two minutes and offers the clearest demonstration of intracellular canaliculi in living material of any tissue that we have as yet encountered. In fact, it offers splendid material for demonstration purposes for classes in cellular anatomy.

Permanent whole mounts of the glands were made by fixing them in Bouin's solution followed by staining in Delafield's or Heidenhain's hematoxylin. These methods were also very useful in the study of sectioned material. For a demonstration of Golgi bodies Nassonov's method proved successful. The mitochondria were revealed by the method of Regaud.

Description

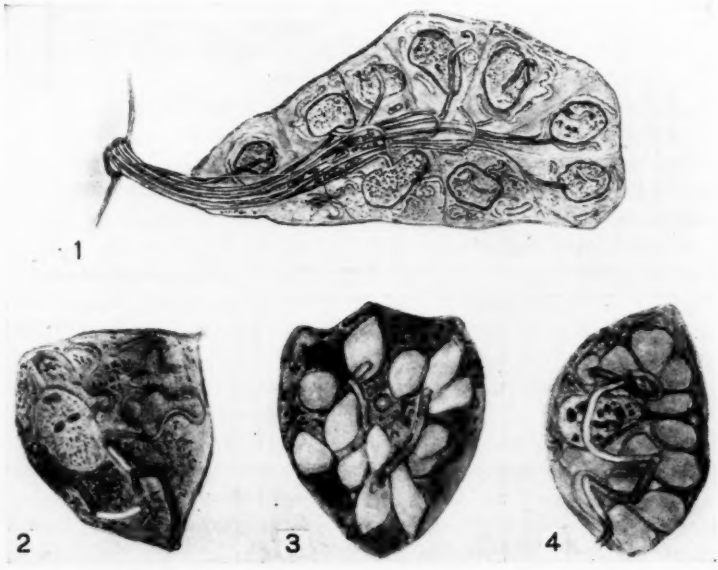
The pharyngeal glands of the worker bee are closely packed about the brain in the upper lateral and anterior part of the head cavity. Each gland consists of a rather long common chitinized duct to which are attached numerous pear-shaped lobules composed of groups of ten to twenty cells. Occasionally lobules consisting of a single cell are found. The lobules extend in all directions from the common duct and each is surrounded by a thin, transparent, yet somewhat tough, membranous capsule which extends as a closed sac from the common duct (Fig. 1).

The cells of the lobules are closely packed together with their bases in direct contact with the enveloping tunica propria. Unlike the lobules of most glands there is present here no lumen, but a central core which contains the chitinous intercellular efferent tubules which lead singly from each cell to the common chitinized duct. Proximally the number of tubules in the core increases as others emerge from the cells of the middle and basal portion of the lobule. Thus the length of a tubule varies with the position of the cell it serves.

These efferent tubules have their source within the cell as simple somewhat sacculated canaliculi without chitinous linings; they take within the cells a rather lengthened sinuous course, coil about the nucleus, and end blindly in the cytosome. Due to this location the ducts are in direct contact with the cytoplasm and when large secretion masses are present they pass through and between them. As the intracellular ducts emerge from the cells each possesses a chitinous lining and finally passes parallel with the others through the enveloping membranous tube formed by the capsule of the lobule (Fig. 1). As the ductules reach the common duct they penetrate it by independent perforations giving rise to papilla-like elevations having, according to Cheshire (1886),

a sieve-like end. Upon careful examination the small nuclei of hypodermal cells, which secrete the chitinous lining, can frequently be observed here and there along the efferent tubules. This chitinous lining apparently serves to keep the delicate tubules open without making them unnecessarily thick and heavy.

PLATE I



1. General topography of a single lobule from the pharyngeal gland of the honeybee. Cells with associated intra- and intercellular canaliculi which lead to the common duct. Whole mount, Bouin, iron hematoxylin.

2. Single cell from pharyngeal gland of honeybee. Intracellular canal and mitochondria. Regaud, iron hematoxylin.

3. Single cell from pharyngeal gland of honeybee. Intracellular canal, secretion globules, and Golgi bodies. Nassonov.

4. Single cell from pharyngeal gland of honeybee. Intracellular canal surrounded by secretion globules. Canal reconstructed from adjacent sections. Champy, iron hematoxylin.

Small globules of secretion are sometimes present in the cells of newly emerged bees. When little secretion is present the globules have a tendency to be located about the nucleus; however, at the end of the secretory cycle they become so large as to mask the cytoplasm almost completely. This secretory cycle has been studied in some detail by Kratky (1931). In such cells the intracellular secretory canaliculi wind

back and forth among and, in some cases, even through the secretion bodies (Figs. 3 and 4). Thus, secretion may pass directly into the canaliculi without traversing great areas of cytoplasm, which would present an effective bar to substances of low diffusibility.

The Golgi material is present in the cells of the pharyngeal gland in its typical insect form, *i.e.*, Golgi bodies with an outer osmiophilic rim or crescent associated with an inner, lighter, and less distinct osmiophobic area (Fig. 3). These are, in general, evenly distributed throughout the cytoplasm and show no indications whatsoever of having any regular morphological relationship to the developing secretory globules. However, in cells packed with secretion, the Golgi bodies are concentrated in the cytoplasm and may, of course, be seen in close but probably fortuitous association with the secretory globules.

The mitochondria have the form of short rods and filaments and, like the Golgi bodies, are distributed fairly regularly throughout the cytoplasm (Fig. 2). Here also there is no morphological evidence of any direct part played by the mitochondria in secretion as has been held by some. The mitochondria are quite distinct from the Golgi bodies and show no signs of transformation from a " passive " to an " active " condition.

Recently, much discussion has arisen regarding the synthesis of secretory products by Golgi material. In fact, the extensive communications of Nassonov and Bowen seem to leave little doubt that the Golgi apparatus is the sovereign structure concerned in the production of secretion. While the contributions of Nassonov, Bowen, and others represent the most careful application of modern cytological technique to secretion, we are not yet convinced that the Golgi apparatus plays an exclusive rôle in the synthesis of all these products. As we have seen from the foregoing description, the Golgi bodies do not in this material show a close topographical relationship to the developing secretory inclusions which would be necessary to establish a direct physiological relationship. To be sure, in many instances a Golgi body is found in direct contact with a developing secretory inclusion, but this, it seems to us, may very likely represent simply a chance position. Furthermore, no great changes in volume of Golgi bodies during the secretory phase are noticeable as might be expected if they directly functioned in the secretion of such enormous globules.

In addition, evidence has been presented by Beams and King (1932*a*) that the limiting membranes of the contractile vacuoles and their associated canals do not represent Golgi material contrary to the views of Nassonov (1923, 1924, and 1925). Nassonov was led to his conception by the fact that these limiting membranes in the contractile

vacuole of certain ciliates blacken in osmic acid and are, according to him, involved in secretory activity. Beams and King have also shown that the limiting membranes of intracellular canals in the grasshopper material studied by them usually blacken in osmic acid, while there is present the usual insect Golgi material. The intracellular canals of the pharyngeal glands of the honeybee do not reduce osmic acid just as in certain ciliates the walls of the contractile vacuole do not. This blackening of structures by osmic acid may be held, then, to be due to their chemical nature rather than to their homology with each other.

Conclusions

1. The pharyngeal glands of the honeybee are composed of lobules which contain ten to twenty unicellular glands.

2. Each unicellular gland possesses an intracellular secretory canaliculus, readily demonstrable in the living condition, which becomes continuous at the apex of the cell with an intercellular chitin-lined duct which joins the common duct of the gland.

3. No evidence was observed to support the theory that the Golgi bodies synthesize the secretory material.

4. In like manner no evidence was observed that the mitochondria directly function in secretion.

5. It is clear that the mitochondria and the Golgi bodies are discrete in the cells of the pharyngeal glands of the honeybee.

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COLOR CHANGES IN FUNDULUS AFTER HYPOPHYSECTOMY

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The pituitary gland is of primary importance in amphibian color change (Hogben, 1924) and its removal from the dogfish produces effects which are similar to those observed in the hypophysectomized frog (Lundstrom and Bard, 1932). In teleosts, however, evidence for any influence of this gland on color change is inconclusive. Certainly in *Fundulus* the activities of the melanophores, and probably of the xanthophores as well (Fries, 1931), are controlled mainly by the nervous system. Nevertheless, recent experiments have shown that other factors, probably humoral in nature, exert some control over the denervated melanophores (Smith, 1931; Parker, 1932) and xanthophores (Fries, 1931), enabling them to respond in an appropriate manner to changes in background though much more slowly than do those pigment cells which remain connected with the nervous system. As Smith points out, these factors may play some part in the activities of innervated pigment cells as well, "probably—furthering the action of the nervous system when the animal remains on the same background for a relatively long period." The nature of the hormone which produces these effects, however, is in doubt. Parker believes that neurohumoral substances are responsible for the changes in tint of denervated areas in *Fundulus*. Smith states that "while in Phoxinus at least humoral factors exert an influence upon the behavior of the melanophores it is at the present moment impossible to enlarge upon this statement," but he suggests the possibility of adrenalin and pituitrin playing a part. Certainly extracts of the posterior lobe of the pituitary do produce definite changes in the pigment cells of both *Fundulus* and *Phoxinus*. Spaeth (1918) found that pituitrin produced a concentration of melanophore pigment when isolated scales of *Fundulus* were immersed in it, and Wyman (1924) obtained similar results with injections of this drug. Hewer (1926) also described a concentration of melanophore pigment and in addition a dispersion of xanthophore pigment following the injection of pituitrin into a minnow, although he is of the opinion that the pituitary gland plays no part in normal color change since the amount of pituitrin necessary to bring

about these changes is, he believes, too large to be produced by the animal itself. In *Phoxinus* the effects of injections of pituitary extracts are apparently different from those observed in *Fundulus*. Abolin (1925) obtained in this fish a dispersion of melanophore, xanthophore, and erythrophore pigments following an injection of infundin and he believed the injection acted in the same way as does the normal reaction to a black background. Similarly, as a result of an injection of infundin, Giersburg (1930) observed a definite dispersion of pigment granules or "expansion" of the melanophores on the sides of *Phoxinus*, together with a pronounced "expansion" of the xanthophores and erythrophores, although he did obtain a "contraction" of the melanophores on the back of this fish. He suggests that the xanthophores and erythrophores and possibly a few melanophores (*e.g.* those on the distal parts of the fins) are controlled by humoral factors, "expansion" of these cells being a result of pituitary activity.

Recently Zondek and Krohn (1932, *a* and *b*) have isolated a substance which they call *intermedin*, from the intermediate lobe of the pituitary of various animals including *Phoxinus*, which, when injected into this fish, produces an "expansion" of the melanophores, xanthophores, and erythrophores. These authors claim that this substance is the only one which will produce this expansion of the erythrophores and that duplication of the red coloration which this fish exhibits during the breeding season can be obtained by injecting a suitable amount of intermedin.

It would appear, then, that humoral factors probably play some part in the color changes of teleosts. Moreover, since the injection of pituitary extracts produces definite changes in color in these fishes, the pituitary gland may be suspected of taking some part in this humoral influence on color change, particularly since this gland is of such importance in color changes of amphibians and its removal has such a pronounced effect on the color pattern of the dogfish. In order to determine what rôle, if any, is played by the pituitary gland in color changes in *Fundulus* the following experiments were carried out.

The hypophysis was removed from a number of killifish (*Fundulus heteroclitus*) in the following way. Each fish was fastened on its back by two strips of wet cloth that were passed across its body and secured to a piece of cork with thumb tacks. A V-shaped cut through the branchiostegal membrane was made with the base of the V at the tip of the tongue. The tongue was then pulled ventrally through the incision which was extended dorso-laterally far enough to expose the region of the hypophysis in the roof of the oral cavity. Usually the hypophysis itself could be seen through the mucous membrane and

bone. The mucous membrane was now cut longitudinally a little to one side of the mid-line (to avoid a median artery) and the tissues loosened from the bone. At first a dental burr was used to penetrate the bone ventral to the hypophysis but this approach left the field so clouded with bone dust that it was difficult to be certain when the hypophysis was completely removed. To avoid this in the majority of cases the bone was cut through with iridectomy scissors and a small window removed, thus exposing the hypophysis which could then be drawn out with forceps or with a fine pipette. The *circulus cephalicus* was frequently cut on one side, but the animals recovered from these operations about as readily as when none of the main arteries had been cut. After the operation no attempt was made to close the incision in the floor of the mouth. The animals were merely kept in N/10 sodium chloride for from 24 to 48 hours and then removed to tap water. In all cases the individuals were examined after death to determine whether or not the hypophysis had been completely removed. Those cases where part of the gland was left behind served as controls. Additional control animals were obtained by performing a number of dummy operations in which the hypophysis was exposed but left intact.

Animals hypophysectomized in this way lived for several weeks, during which time their reactions to background changes were examined. As usual a number failed to react, a common experience even with unoperated animals. The majority of both the hypophysectomized and control animals, however, reacted exactly as do normal animals when placed over white, black, or yellow backgrounds, becoming light over white, dark over black, and a decided yellow color over the yellow background. Microscopic examination of the tail fins of these animals showed that, as usual, the melanophores were contracted over white and yellow backgrounds and expanded over black, while the xanthophores were contracted over white and expanded over yellow. Many of the animals became lighter in color a few days after the operation due to degeneration of some of the melanophores, as could be seen when a scale or the tail fin was observed under the microscope. But here again no difference could be detected between hypophysectomized and control animals, hence the bleaching of these animals could not have been due to the absence of the hypophysis.

Parker and Lanchner (1922) have shown that when a *Fundulus* is put in total darkness it becomes light due to contraction of its melanophores. This reaction was also tested. When a light-proof box was placed over the aquarium in which a hypophysectomized animal was

swimming, the removal of this hood an hour or so later showed that the fish had become definitely lighter in color, again behaving like a normal animal.

In order to determine whether or not the pituitary body is concerned with the slow changes in tint of denervated areas which Smith (1931) and Parker (1932) have observed, the following experiments were performed. A transverse cut was made in the base of the tail of six animals following the technique described by Fries (1931). A black area extending distal to the cut gave evidence that the nerves running to the melanophores in this area had been severed. The hypophyses were then removed from three of these animals, the other three serving as controls. When kept continuously over a white background it was found that the denervated melanophores of the tail contracted at approximately the same time in both hypophysectomized and control animals, about three days being required to complete the change in both cases. Similar results were obtained for denervated areas of the trunk. Although the reaction here was not as definite as that in the tail, nevertheless black denervated areas of the trunk did become smaller after several days over a white background in hypophysectomized as well as in control animals. Obviously removal of the hypophysis does not prevent denervated areas from changing in tint when the fish are transferred from one background to another.

It is apparent from these experiments that the removal of the hypophysis from *Fundulus* does not affect the ordinary reactions which it exhibits to changes in background or to total darkness. But since commercial posterior lobe extracts do cause the melanophores of this fish to contract (Spaeth, 1918; Wyman, 1924), the question naturally arises as to whether or not the pituitary body of *Fundulus* contains any of the melanophore principle. The pituitary bodies of other fish have been shown to contain this principle. Hogben and Winton (1922) found that extracts of cod pituitary produced the usual effects when injected into frogs, and Zondek and Krohn (1932*b*) have shown that "intermedin" can be extracted from the pituitary of *Phoxinus*. The following experiment shows that the hypophysis of *Fundulus* also contains the melanophore principle. The pituitary, forebrain, medulla, and a small piece of trunk muscle were removed from a *Fundulus* of average size (6 to 8 centimeters in length) and separate extracts were made of each by grinding them in 0.05 cc. of N/10 sodium chloride solution. As test objects a number of scales from another fish were mounted in hanging drops of the same medium in which, as usual, the xanthophores contracted and the melanophores expanded. When the fluid on one of these scales was exchanged for the brain or muscle

extracts no change was observed in the pigment cells In fact, when scales with contracted melanophores and expanded xanthophores were mounted in these brain extracts, the melanophores would expand and the xanthophores contract, the brain extract thus having the same effect that ordinary sodium chloride solutions would have. But when a scale preparation was changed to the pituitary extract, the xanthophores always expanded, while the melanophores contracted. Obviously these extracts of the pituitary body were crude and probably only a small proportion of the active principle or principles of the posterior lobe could be removed in this way (Kamm et al., 1928). As a matter of fact, the reaction of the pigment cells was slower than that produced by N/10 KCl, for example, as several minutes were required for the maximum effect to be reached and even then the melanophores were not always contracted to a punctate condition. Moreover, the effect of the pituitary extract lasted for only about 45–60 minutes, after which the melanophores slowly reëxpanded, and although they could be made to contract again by adding the extract a second time, this second contraction was rarely as complete as the first. Nevertheless, these experiments show that the pituitary body of *Fundulus* does contain a substance which will produce an expansion of the xanthophores and a contraction of melanophores in isolated scale preparations. How this fact is to be reconciled with the results described above, namely that the removal of this gland has no apparent effect on subsequent color changes of the animal, is not evident from these experiments. It is possible, as Hewer (1926) suggests, that any melanophore principle formed by the hypophysis is liberated in quantities too small to have any effect on the pigment cells. However this may be, it is clear from the experiments first described that the removal of the hypophysis from a *Fundulus* does not affect the ability of that animal to change its color in apparently the same manner as does a normal animal.

Summary

When the hypophysis of a *Fundulus* is ground up in 0.05 cc. N/10 NaCl and a scale from this fish is mounted in such an extract, the xanthophores "expand" while the melanophores "contract," although brain and muscle extracts prepared in the same way have no effect on the pigment cells. The hypophysis of *Fundulus* does contain the melanophore principle. In spite of this fact, when the hypophysis is removed from *Fundulus*, the animal still responds to changes in background and to total darkness in the usual manner. Moreover, the slow change in tint of denervated areas occurs as readily in hypophysectomized as in normal animals. In other words, the removal of the

hypophysis has no apparent effect on the pigmentary responses which *Fundulus* may exhibit.

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A NOTE ON AMPHIOXIDES FROM BERMUDA BASED ON DR. W. BEEBE'S COLLECTIONS

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The "Challenger" Expedition caught on the high seas a peculiar pelagic branchiostomid to which later the name of *Amphioxides* was given by Gill. Our knowledge of these forms dates from the author's monograph (1905) based on the material of the "Valdivia" Expedition. The interesting anatomy was thoroughly studied, resulting in the discovery of whole organ-systems not yet known in the Acrania, viz. the visceral musculature, the musculature of the mouth, the canal-system of the head region, connective tissue and skeletal system, the nephridial nature of the so-called Hatschek's nephridium, discoveries which have since all been verified for *Amphioxus*. The question which then arose was whether these *Amphioxides*-forms represented a family of pelagic Acrania or whether they were peculiar larval forms. In his first monograph the author decided in favor of the first view, because all *Amphioxides* were caught far away from the shore and mostly in considerable depth; also because up to 35 gill-slits specimens were found without a trace of metamorphosis; and finally because some of the largest specimens had already nicely developed gonads. Of course the possibility was discussed that larvæ of some species of Acrania were carried out to the high seas and became there neotenic. Only one year later (1906) the author had to accept the latter alternative because he received from the North Seas a larva of the ordinary *Branchiostoma lanceolatum* which had not undergone metamorphosis at the usual stage but grown into an *Amphioxides* larva. A few years later (1909) the author was able to study still other material which yielded further information. The new material did not come from the deep sea but from the surface. The decisive fact was the presence of a number of individuals, taken off the mouth of the Amazon river and belonging to the species *A. valdiviae* Goldsch. which had begun with metamorphosis at stages with 25–34 gill-slits. This find, of course, settled the larval character of the *Amphioxides* forms. In 1910 appeared a paper by Gibson on the basis of the material of the Percy Sladen Trust Expedition, which had collected the largest amount of *Amphioxides* material yet known. Besides a few minor discrepancies in details of anatomy, some of which have since come out in favor of

my description, the author adds the fact that besides catches in the deep sea one very large haul of *Amphioxides* was made near shore in a region inhabited by *Asymmetron lucayanum*. Though the anatomical details of the two forms did not agree completely, Gibson holds that *A. pelagicus* is the larval form of *Asymmetron lucayanum*. (My last-mentioned paper, reporting the metamorphosis, was not yet known to the author.)

TABLE I

Dr. Beebe's material consisted of 87 specimens, of which 73 were available. When number of slits or length is not stated, the condition of the specimen did not permit an exact statement.

Haul No.	Depth	Date	Remarks	Number
	meters			
253	1829	4/7/29	7 mm., 20 slits	1
357	1463	9/8/29	Macerated	1
363	1829	10/8/29	11 mm., 22 slits	1
386	1280	17/8/29	14 mm., macerated	1
400	1829	31/8/29	All ca. 8 mm., 18–22 slits	41 (27)
401	2011	31/8/29	Macerated	1
404	1097	2/9/29	Ca. 10–12 mm., badly preserved	11
406	1463	2/9/29		
407	1646	2/9/29		
498	7	23/9/29	Macerated	1
519	0–183	30/9/29	Typical	1
524	0–1097	30/9/29	Typical	1
735	1829	27/6/30	23 slits	1
761	1646	2/7/30	Macerated	1
791	914	9/7/30	Macerated	1
818	1097	29/8/30	15 mm., 26 slits	1
835	914	3/9/30	Macerated	1
875	1097	11/9/30	Macerated	1
907	0–183	18/9/30	Typical	3
909	0–549	18/9/30	21 slits	2
910	0–732	18/9/30	8–10 mm., 24 slits	7
912	0–914	18/9/30	22–26 slits	3
913	0–1463	18/9/30	14 mm., 24 slits	1
1063	549	8/7/31	20 slits	1
1193	914	17/8/31	Macerated	1
1301	92	15/9/31	21 slits	1
1344	0	3/11/31	20 slits	2

Though we know now that *Amphioxides* is a larval form, able to metamorphose at an uncommonly late stage, we do not know whether this is the ordinary type of development of certain forms like *Asymmetron*. After more than twenty years I was therefore much gratified to be able to return to this old topic during a stay at the Bermuda Biological Station. Dr. William Beebe was kind enough to let me have all the *Amphioxides* which he had caught near the Bermuda

Islands during his expeditions for the New York Zoölogical Society, and the Director of the Station, Dr. Wheeler, gave me the opportunity to compare this material with the living *Asymmetron* of this region. I should like to thank both gentlemen for all courtesies. The point in question was whether the *Amphioxides* forms near the Islands could be shown to coincide with the native *Asymmetron lucayanum*.

The *Amphioxides*-material was caught about five miles off the coast of Bermuda Islands with deep sea nets,[1] the contents of which were

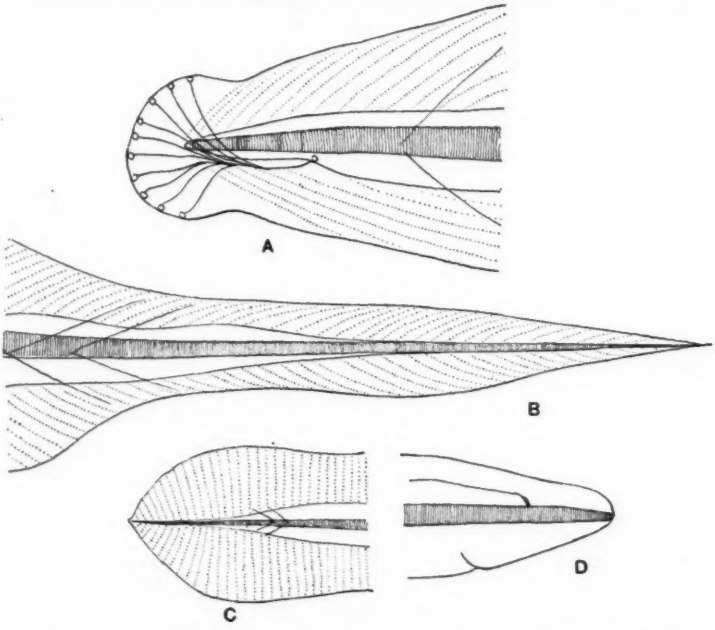

FIG. 1. *A*. Outline sketch of head of *Asymmetron lucayanum* from Bermuda. *B*. Tail of same. *C*. Outline sketch of head of *Amphioxides pelagicus* from Bermuda. *D*. Tail of same.

supposed to have been caught mainly at the depths stated in Table I. But whereas no closing net was used, these data are not beyond criticism. They agree, however, with the data of former work which show the occurrence of *Amphioxides* from the surface down to rather considerable depths. In the majority of instances only one or a few individuals are reported. But one haul netted 41 specimens at once,

[1] For details see Beebe, W., 1931. Bermuda Oceanographic Expeditions *Zoölogica*, 13.

an occurrence which is paralleled in the material of the British Expedition. The size of the specimens varies between 8 and 16 mm. All of them belong to the same species, namely, *A. pelagicus*. The gill-slits do not increase regularly with size. It seems that at a stage of ca. 8 mm. almost the maximum number of gill-slits is already formed and only a few may be added afterwards. At this stage 18–22 slits are found and the largest individual possessed only 26. This again agrees with former findings. The decisive systematic character for comparison with other forms is the number of segments. In all suitable specimens I counted 50–51 pre-anal and 12 post-anal segments. This count does not agree with the typical *A. pelagicus*, which has 53 pre-anal and 14 post-anal segments (see later).

The typical *Asymmetron lucayanum* of the Caribbean has, according to literature, 53 + 13 = 66 segments. In the Bermuda form I have counted 67 segments which would agree with the Pacific *A. pelagicus* but not with the Caribbean. After the segmentation the most characteristic feature of *A. lucayanum* is the form of the snout and of the tail-fin. Both are absolutely different from the *Amphioxides* forms, as the figure shows. The alternative conclusions then are: either the Bermuda *Amphioxides* do not belong to *Asymmetron* or they are able to change their shape and segmentation completely during metamorphosis. The latter seems rather improbable.

There also exists in the Bermudas a species of *Branchiostoma*, *B. bermudae* Hubbs. But its myotome number is only 54–57, which excludes any relation to the *Amphioxides*. The relation between *Amphioxides* and the *Amphioxus*-forms of the nearest littoral region is therefore unsettled in this case also.

The actual situation then is:

1. *Amphioxides* forms are found all over the world (see map in Goldschmidt, 1909).

2. At least three clearly distinct types can be distinguished, namely *pelagicus* Gill, *valdiviae* Goldschmidt, *stenurus* Goldschmidt.[2] Probably *pelagicus* consists of more types (*see* the Pacific form with 67 segments, the Bermuda form with 62–63, and the North Sea form certainly belonging to *B. lanceolatum* Pallas).

3. *Amphioxides* forms are found in the littoral zone (one case of Gibson) and on the high seas from the surface to the deep sea.

4. *Amphioxides* forms grow and differentiate far beyond the normal stages of metamorphosis found in the littoral zone.

5. They are able to metamorphose in far advanced stages of growth and differentiation, as shown for *A. valdiviae*.

[2] Not *sternurus*, as Gibson and Hubbs write,—stenos = narrow.

6. Occasionally the development of gonads begins before metamorphosis and proceeds rather far.

7. In those cases which are known thus far, only right gonads were formed, which is typical for the genera *Epigonichthys* and *Asymmetron*.

8. Thus far no *Amphioxides* could be assigned with absolute safety to a neighboring littoral species with the exception of the specimen from the North Sea belonging to *Branchiostoma lanceolatum*.

After taking all these facts together I come to the following conclusions: It seems that all *Amphioxi* have (always or under certain conditions?) two types of larvæ: one metamorphosing *in loco* and continuing the community in a given littoral region; another leaving the littoral for a more or less prolonged pelagic life with a much later metamorphosis, thus enabling the spread of the species. Biologists living in an *Amphioxus* region ought to be able to test this suggestion.

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DIURNAL CHANGES IN ACTIVITIES AND GEOTROPISM IN THYONE BRIAREUS

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I

In *Thyone briareus* waves of constriction completely encircling the body and involving both bivium and trivium travel along the longitudinal axis of the body. These waves usually serve as a means of locomotion and aid in extruding the tentacles. However, at times waves course the whole length of the body without producing any progression of the animal. Whether waves pass toward the oral or aboral pole depends upon the presence of certain forces in the environment of the animal and upon the incidence of periods of activity in *Thyone*. The following experiments, investigating the control of the locomotor waves, were made at the Marine Biological Laboratory, Woods Hole, during January, 1928, and since then have been repeated at various times in the Laboratory of General Physiology, Cambridge.

II

At 16.0° C. (7:00 P.M., in winter) a new wave of constriction originated at one pole about every four minutes (4.3 minutes average) and progressed with a uniform speed (0.5 mm. per second at 16.0°; 0.9 mm. per second at 25.5° C.) toward the opposite end. The rate of progression was practically the same in either direction (oral to anal, 0.87 mm. per second average; anal to oral, 0.89 mm. per second at 25.5° C.). During the morning and early afternoon few locomotor waves were seen, also fewer animals extended their tentacles. Beginning at about 4:00 P.M. (winter months, Woods Hole) more waves were seen and more animals had their tentacles extended. The same periodicity of activity was noticed even when the animals were kept in a dark room and viewed by red light. This state of increased activity continued until about 1:00 or 2:00 A.M.; thereafter the animals exhibited fewer locomotor waves and the tentacles remained retracted.

When the tentacles were not extended the waves of constriction usually originated at the oral end and coursed toward the anal region; when the tentacles were extended the waves started at the anal end and traveled toward the oral pole. It is as if the waves originated at that pole exhibiting the lesser activity. When the oral end is quiet the anal end is, of course, relatively more active since at that end the respiratory movements go on all of the time.

While it is usual to find at any moment only one wave traversing the animal, two waves have been seen in some instances going in the same direction; occasionally two waves were seen going in opposite directions. Sometimes waves were seen to originate simultaneously at both ends of the animal and approach each other. At the meeting point, one of the waves disappeared; the other one continued its course to the anal end. Such phenomena frequently occur in animals possessing a nerve net. (Mayer, 1906, 1908; Crozier, 1915; Child, 1917. In the intestine of mammals, Alvarez and Mahoney, 1924.) Apparently in *Thyone* there are at each end of the animal regions initiating the onset of locomotor waves.

While the animal is in a horizontal position, waves can be started experimentally at almost any place along the longitudinal axis of the body by producing a tension in the selected region. All experiments were made in the evening. (1) A small region of the bivium of animals which had attached themselves to a substratum was loosened and a glass plate of 1 cm. thickness and 1 cm. width was slipped beneath the animal. (2) A 45° glass prism was placed under the animal so that it fastened itself over the angular edge of the prism. (3) A hæmostat was attached to the tube feet of the trivium and the animal was freely suspended in sea water in a horizontal position. (4) *Thyone* was placed in a beaker small enough to cause the animal to take up a U-shaped form. In all these experiments the waves of contraction appeared at the site of stretch or tension. This was also found to be true for *Thyone* which had recently eviscerated.

A local tension brings about a contraction which then travels toward one or the other end of the animal. Experiments by Henderson (1928) on peristalsis of the guinea-pig and rabbit intestine show that nerve cells arranged in short reflex arcs are concerned in the peristaltic mechanism of this organ. Slight increments in pressure were found sufficient to elicit a peristaltic wave. Cannon (1911, p. 187) concluded from his investigations on the same question that the muscle fibers which are stretched tend to contract. The basic rhythm of contraction in the intestine seems to have a myogenic origin (Alvarez and Mahoney, 1922).

Work by Tao (1927) suggests that the contraction of smooth muscle in holothurians is controlled myogenically. The appearance of locomotor waves in *Thyone* is similar to the beginning of peristaltic waves in the stomach, where the pacemaker controlling the frequency of appearance of waves seems to be located in the region on the lesser curvature next to the cardia (Alvarez, 1928, p. 106). In *Thyone* it is as if a pacemaker mechanism is controlling the formation of a new wave every four minutes (at 16° C.) at either the aboral or oral end. However, as was shown above, the waves can be initiated experimentally by the production of an appropriate tension in the body wall. It is still unknown whether the locomotor wave originates at one end of the animal and first makes its appearance as a constriction at the locus where the tension was applied, or whether the wave of contraction originates at the locus of tension. Alvarez (1928, p. 108) has recorded many instances where a wave is seen to originate at the cardiac end of the stomach, disappear from view entirely for a short distance, and then reappear at a region caudad to the origin. A few of my observations on *Thyone* agree with these findings of Alvarez. The animal was placed in a beaker so as to take up a U-shaped form; the waves originated at the locus of bending, travelling sometimes to the oral end, sometimes to the aboral end. When the tentacles were withdrawn the waves travelled aborally, when the tentacles were extended the waves travelled orally, starting in all cases from the locus of bending. One might think of the wave of contraction as originating at the pole exhibiting the lesser activity but not becoming visible until the region of tension was encountered. Very often waves of contraction starting at one end of the body suddenly faded out further along the axis of the animal. Whether the initiation of waves of constriction at either pole of the animal (when it is in a horizontal position) is controlled by tensions developed in these regions or whether an oral or aboral pacemaker sets off the wave of contraction is still an open question.

III

During the morning and early afternoon the origin of progression waves in *Thyone* is usually fixed at one of its poles (oral), regardless of which end pointed down. The tentacles were usually retracted during this part of the day. Such persistence of polarity continued until about 7:00 to 8:00 o'clock in the evening. When *Thyone* was then placed in a vertical position the waves were found to begin at the bottom, regardless of which end of the animal pointed down, and coursed upward and moved the animal in that direction. Under these experimental condi-

tions *Thyone* can be thought to exhibit negative geotropism. After 1:00 A.M. the polarity remained fixed, usually at the oral pole. In these experiments glass plates to which the animals had attached themselves while in a horizontal position were rotated so as to bring the body axis into a vertical position. In alternate tests aboral and oral poles were placed at the bottom. In other experiments this procedure was repeated with V-shaped glass troughs; also with glass tubes. Even when the animal was suspended from either end by a small hæmostat attached to several tube feet, the waves continued their upward movement whether the oral or aboral pole was placed at the bottom. No mention of this curious diurnal alteration in the locus of origin of the waves was made by Pearse (1908) in his observations on *Thyone*.

Negative geotropism was only obtained regularly during the midwinter months. The reversal of polarity of locomotor waves when the animals were rotated in the gravitational field was only occasionally obtained during the months of May, June, July, and August at Cambridge; during this time of year *Thyone* did not show a regular diurnal periodicity of activity.

The reversal of direction of the locomotor wave on the body of the animal according to the orientation with respect to gravity seems to give an instance of geotropic stimulation in which the structural polarity of the organism is of secondary consequence. Emphasis is placed upon the directional control of the wave movements, rather than upon locomotor progression of the animal, because the waves may be obvious without actual creeping.

A reversal of upward, geotropically-oriented creeping of tree snails such as *Liguus, Pleurodonte,* and others, has been brought about by Crozier and Navez (1930). The upward orientation was shown to be governed by the sensible equality of tensions produced through the pull of the body upon its supporting elements. Downward orientation on a vertical surface was simply obtained by properly adjusting the pull upon the shell (by a string attached to the shell or by a load of 5 grams attached to the shell). Under water *Liguus* also orients upward. Reversal of orientation under water on a vertical surface can also be obtained by attaching a cork float of appropriate size to the apex of the shell. In both these cases of reversal of orientation and in additional experiments on geotropic excitation in *Helix* (Hoagland and Crozier, 1931–32) it is difficult to conceive a mechanism dependent primarily upon a statocyst. These facts are entirely consistent, however, with the notion of proprioceptive stimulation through impressed tensions as the controlling feature in the gravitationally excited orientation (*cf.* Crozier, 1929).

Evidence presented above points clearly toward a deficiency of statocyst control of geotropism in Thyone, since no specific polarity is encountered in upward creeping. In fact, in an aquarium, equal numbers of the animals will be found with either pole uppermost. This was also observed by Pearse (1908). The absence of statocyst function is further suggested by experiments with eviscerated animals, which still can be made to exhibit negative geotropism during the late evening hours in the winter. In such animals the tentacles, the stone canal with its nerve ring, and the gut are thrown out in their entirety; the possibility of attributing the origin of these waves to tension on the mesentery of the viscera, as has been shown to be the case in the righting movements of starfish (Wolf, 1925) and as suggested by Parker (1922) for the orientation of the sea urchin *Centrechinus*, seems to be eliminated. The cloaca and the attached trachial trees have also been removed from eviscerated animals and they still always oriented upward, during the evening hours of winter months.

When the holothurian *Caudina chilensis* is imbedded in sand and is rotated (Yamanouchi, 1929), it responds to centrifugal force in such a manner that the oral end bends away from the center of rotation and the tail turns toward the center of rotation. Even when the anterior part of the body including the nerve ring is amputated the tail reacts to centrifugal force in the same manner as the intact *Caudina*. Statocysts are apparently not involved in these reactions. The responses seem to be brought about by differential tensions in the body wall produced by the rotations. The evidence from experiments on *Synaptula* (Olmstead, 1917) and *Cucumaria cucumis* (Loeb, 1891) suggests that a statocyst mechanism orients the animal so that its oral end is always upward while ascending a vertical plane by aid of its tentacles. Clark (1898, 1899) has described structures in the oral stone canal of *Synaptula* to which he has assigned the function of statocysts. No mention was made in these studies of any diurnal fluctuations in the animal's response to gravity, so we assume that it remains negatively geotropic throughout the entire day.

The sensitivity of the body wall of *Thyone* is so altered diurnally that the structural polarity of the body is of minor consequence when the animal is oriented by geotropic excitation. The deforming pressure of the pull of the body wall when the animal is on a vertical plane elicits a locomotor wave at its lower end. The direction of the waves is primarily determined by the force of gravity only during the evening, and then especially during the winter months; only at this period of the day

and year have waves been found to originate at either pole and course upward.

Summary

Thyone briareus observed during the winter months exhibited certain definite diurnal periods of activity. (1) Between about 4:00 P.M. and 1:00 to 2:00 A.M. the frequency of "locomotor" waves increased in number and the feeding movements of the tentacles were more numerous than at other periods of the day, even when observed in the dark under red illumination. (2) When during the late evening hours *Thyone* was rotated in a vertical plane, locomotor waves coursed negative to gravity regardless of which end of the animal was oriented downward. Since it was found that waves of contraction could be elicited experimentally at almost any region by producing tensions in the body wall when *Thyone* was in a horizontal position, it is supposed that the sensitivity of the body wall is so altered during the late evening hours that tensions produced in the body wall when the animal was placed in a vertical position are sufficient to bring about the formation of locomotor waves at the lower end. Absence of statocyst function is suggested by these experiments, and by additional experiments with eviscerated animals which still can be made to exhibit negative geotropism.

During spring and summer months the activity of *Thyone* was not so definitely marked off into diurnal periods, neither was the negative geotropic response regularly obtained.

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MONOCENTRIC MITOSIS WITH SEGREGATION OF CHROMOSOMES IN SCIARA AND ITS BEARING ON THE MECHANISM OF MITOSIS[1]

I. The Normal Monocentric Mitosis. II. Experimental Modification of the Monocentric Mitosis

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I. The Normal Monocentric Mitosis

It has become a generally accepted principle in cytology that a prime essential for complete mitosis is the presence of a bipolar or multipolar spindle (cf., *e.g.*, Wilson, 1925, p. 168; Gray, 1931, p. 172) and that in a unipolar field, although the chromosomes may divide, they cannot segregate into two groups. In flies of the genus *Sciara*, however, the first spermatocyte regularly undergoes as a normal process a monocentric mitosis which effects a regular and precise segregation of chromosomes (Metz, 1926*a*; Metz, Moses, and Hoppe, 1926). Since this process deviates widely from that observed in ordinary mitosis it has invited careful analysis, and has led to a series of studies involving several species.

Occurring as it does in the reduction division, the mitosis in question segregates homologous chromosomes. Consequently genetic as well as cytological methods have been used to trace the behavior of individual chromosomes (Metz, 1926*b*, 1927, 1928, 1929), and to determine the principle underlying the segregation process (see below). The present brief account is concerned primarily with evidence bearing on the mechanism of chromosome movement.

In the prevailing theories of mitosis[2] there has been a strong tendency to attribute the movement of the chromosomes to the poles in anaphase to forces of attraction or repulsion, or both, emanating from outside the chromosomes, and to consider the chromosomes themselves

[1] The second part of this investigation has been aided by a grant from the National Research Council, Committee for Research on Problems of Sex. The writer is indebted to Mrs. Philip B. Armstrong (M. Louise Schmuck) for assistance in that part of the investigation.

[2] See, *e.g.*, Wilson, 1932; Sharp, 1926; Bělař, 1929*a*, *b*, *c*; Gray, 1931.

as playing a relatively passive rôle, or at most as exerting a mutual repulsion.

So far as I am aware, no significant indications have been observed in ordinary mitoses that autonomous activity on the part of the chromosomes themselves plays an important part in their anaphase movements,[3] although various lines of evidence suggest that they may exhibit such activity at other stages.[4]

In the monocentric mitosis in *Sciara* the anaphase movement of certain chromosomes exhibits characteristics which strongly suggest that it is due in large part to the activity of the chromosomes themselves. These characteristics will be considered briefly below.

As noted in the papers referred to above, the monocentric mitosis in *Sciara* also presents evidence bearing on the much-debated question of the rôle played by the so-called " spindle fibers " in mitosis. It indicates that, whatever their precise nature may be, they represent a functional reality and reflect an activity operating on the chromosomes in the direction of the pole. (The active agent here may be the " insertion region " of the chromosome, or may be external to the chromosome.)

In the present studies observations have been made on both sectioned material and smears. In all cases the testes were dissected out and freed from surrounding material before fixation. On account of their small size, this permitted almost direct contact of the cells with the fixing fluid even in the case of the sectioned material. In the smears the cells were, of course, brought immediately into direct contact with the fixative. Excellent fixation was secured in both ways. Various fixatives have been used, but most of the observations have been made on material fixed in Gilson's mercuric-nitric mixture and stained in Heidenhain's iron haematoxylin. Flemming's fluid has been used, but with unsatisfactory results due to the presence of large chondriosomes which obscured the mitotic figure in most cells (see Metz, Moses, and Hoppe, Fig. 34). At the stage in question these chondriosomes usually form a saucer-shaped or cup-shaped mass lying peripherally and often extending about halfway around the cell. They have received special study by Mr. W. L. Doyle (paper in press).

Unfortunately details of the achromatic figure are not as satisfactorily brought out after fixation with Gilson as by other methods, and for this reason certain of the finer aspects of the figure have not yet been analyzed. The features essential for present purposes, however, are re-

[3] Since this was written, papers by Bleier (1931) and by Wilson (1932) have come to hand, both emphasizing the possibility of such autonomous activity. The literature on the subject is reviewed by both authors.

[4] See, *e.g.*, McClung, 1927; Lewis, 1932; Lewis and Lewis, 1932.

vealed clearly enough in the sections studied. In the smears the achromatic figure is so exceedingly delicate in structure as to be almost invisible, due presumably to the delicacy of the fixation and the similarity of the refractive indices of the materials in the figure. The general nature of the structure, however, is clearly indicated by the configuration and behavior of the chromosomes.

All the material is from pedigreed cultures which have been kept in the laboratory, in some cases for many generations.

The principal characteristics of the monocentric mitosis in *Sciara coprophila,* Lint. may be summarized as follows: (For details see Metz, Moses, and Hoppe, 1926.)

1. The spermatogonial chromosome group as shown in Text Fig. 1 typically consists of ten members: three pairs of rod-like chromosomes, one pair of small V-shaped chromosomes, and one pair of much larger V-shaped chromosomes (the " limited " chromosomes). The " limited " chromosomes are limited to the germ-line, due to elimination from somatic nuclei in early cleavage.[5]

2. No synapsis occurs. In prophase of the first spermatocyte division (Text Fig. 1) the univalent chromosomes appear to be distributed at random, but about equidistant from one another, about the periphery of the nucleus when the nuclear membrane breaks down.

3. After the nuclear wall vanishes a half spindle appears and the chromosomes, apparently without changing their locations, all become oriented toward the single pole, each with a " spindle fiber " extending from the normal insertion point on the chromosome toward the pole. No centrioles have been identified.

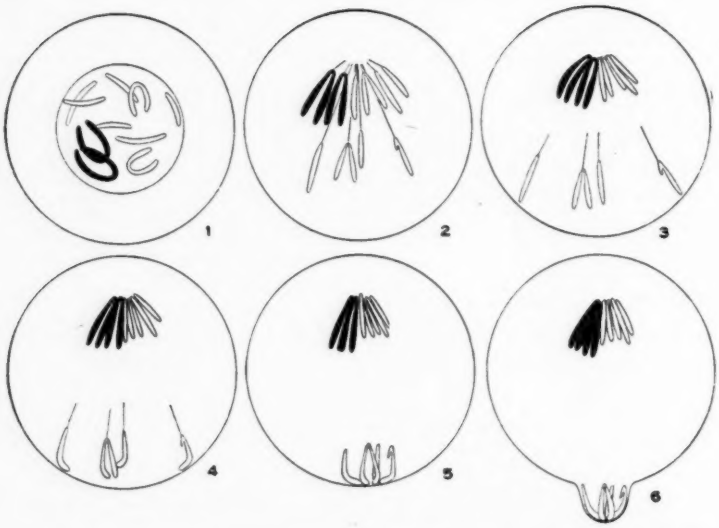
4. The chromosomes do not become arranged in any one plane and nothing comparable to an equatorial plate or metaphase stage is found. On the contrary, they move from their prophase positions either directly toward, or directly away from, the pole (Text Fig. 2).

5. Certain chromosomes regularly move toward the pole in the customary fashion. These include both of the large " limited " chromosomes and one member of each of the other four pairs of chromosomes. Genetic evidence indicates that among the latter four pairs it is regularly the maternal member which passes to the pole. This evidence appears to be complete for at least three of the four pairs in one species.[6]

6. The remaining four chromosomes—homologs of four which go to the pole—regularly move " backward " directly away from the pole, following radial, and hence diverging, paths as far as possible from the common center. When the chromosomes reach the periphery of the cell their courses are deflected and made to converge, ultimately bringing

[5] See Metz, 1931; DuBois, 1932; Schmuck and Metz, 1932.

[6] Metz, 1927 and unpublished evidence of Dr. Helen B. Smith and the writer.



TEXT FIGS. 1–6. Schematic representation of the movements of the chromosomes in the monocentric first spermatocyte division in *Sciara coprophila* Lint.

Diagram 1. Prophase just before dissolution of the nuclear membrane. The chromosomes are scattered, apparently at random, about the periphery of the nucleus.

Diagram 2. Beginning of migration of the chromosomes—six toward the pole and four directly away from it.

Diagram 3. Slightly later stage, after the six chromosomes have reached the pole and the other four have progressed to the periphery of the cell. The two median "retreating" chromosomes are presumed to be at high and low levels, respectively, and at the periphery.

Diagrams 4–6. Successively later stages showing the migration of the chromosomes along the periphery of the cell away from the pole, and the beginning of the extrusion process at the point farthest from the pole.

Actually the paths of the retreating chromosomes are only rarely equidistant from one another as shown in these diagrams (see text), and the chromosomes which pass to the pole do not ordinarily retain their polar orientation later than the stage shown in Diagram 3.

The two "limited" chromosomes are represented in black. These regularly pass to the pole and are retained. Among the other chromosomes precise segregation of homologs is effected. The hook at the proximal end of the retreating chromosome at the right, due to sub-terminal fiber attachment, is presumably characteristic of its homolog also, but is not evident in the latter, probably because of the lack of stretching. As will be seen from the camera drawings (Plate I), the retreating chromosomes are comparatively more elongate than the diagrams indicate. (See also Figs. 14–18 in Metz, Moses, and Hoppe, loc. cit.)

them together at a peripheral point opposite the single pole. Subsequently they are extruded in a finger-like process, where they degenerate (Text Figs. 3–6).

7. During their "backward" movement the four chromosomes retain their reversed orientation, moving with the spindle fiber insertion point hindmost, and the free end, or ends (in the case of the V-shaped member) foremost.

8. The posterior portion in these chromosomes is commonly taut and slender and often almost detached from the remainder, giving convincing evidence of a force, represented by the "spindle fiber," operating in the direction of the pole and in opposition to the movement of the chromosome (see Figs. 7 and 8).

9. In contrast, the apical ends of these chromosomes are thick, usually curved or twisted, and lie free, with no indication of any traction operating on them (see photographs, Plate I). It is clear that they are not being "pulled" in the direction of movement.

It is thus possible to distinguish the operation of two forces on the four retreating chromosomes. One is a retarding force, represented by the so-called "spindle-fibers" operating in the direction of the pole and only on one region of the chromosome, the insertion point. The other, and superior, activity operates in the opposite direction, and involves the chromosome as a whole, serving to cause the movement described above. It is the latter activity which seems to be performed mainly by the chromosome itself rather than by an outside agent.

As noted previously (Metz, 1926*a*), the chromosomes, during the movement in question, give the appearance of being carried by currents in the protoplasm. But careful study of a large amount of material prepared in various ways has convinced me that no true currents are involved and that the only flowing motion concerned is that of the material immediately around the individual chromosome. The details of the process will be presented in another paper. They may be summarized, however, by saying that each of the retreating chromosomes moves off independently, away from the pole, either in a small island of material (gel?) from the original half-spindle, or at the apex of a pseudopod-like outgrowth of this material.

Some idea of the independent movement of these chromosomes, and of the general aspect of the figures, may be gained from the drawings shown in Figs. 7, 8, and 10 and from the photographs in Figs. 15–17. For descriptions of these see explanation of figures.

That the movement is due to an activity of the chromosome itself is suggested mainly by two features. The first of these is the independent action of the individual chromosomes, each of which not only follows its

own course, but follows a course apparently determined only by the original position of the chromosome in relation to the pole. This original position appears to be determined at random. If two or more of the four chromosomes happen to be adjacent at the beginning they move away together and follow essentially the same path. If they are well separated at first their paths diverge widely (see, *e.g.*, Fig. 17). Furthermore, the path of any individual away from the pole may follow any radial line within the theoretical cone delimited by imaginary extension of the half spindle. In other words, it seems impossible to explain the phenomenon on the basis of predetermined currents or lines of flow in which the chromosomes are caught and transported.

The second feature concerns the position of the chromosome in the island or process of " spindle " substance in which it moves. This position is not easily determined with accuracy, for only occasionally is the outline of the mass revealed. However, numerous large chondriosomes and other stainable constituents present in the cytoplasm sometimes delimit the clear mass distinctly. Careful study of such cases indicates that typically the position of the chromosome is apical. Since, as already noted, there is clear indication that the movement of the chromosome is retarded by an activity represented by the " spindle fiber," it seems improbable that the chromosome would remain at the apex of the moving mass unless it were itself responsible for the movement. In other words, were the chromosome simply being transported by an outward flow of the material from the half spindle, the retarding " spindle fiber " force would presumably cause it to move less rapidly than the surrounding material and hence not remain at the apex of the outflowing mass.

As a result of the above considerations and others of a more detailed nature, the hypothesis is advanced that the retreating chromosomes move because of their own activity. It may further be postulated that this activity operates by bringing about a progressive alteration in the physical state (*i.e.*, by solation or gelation) of the protoplasm adjacent to the chromosome.[7] Presumably the simplest form of such activity would be one producing a change in front of or behind the chromosome—the former serving to pull the chromosome along, so to speak, and the latter acting more as a pushing agent. Without attempting a detailed discussion of such possibilities, it may be noted in this connection that the

[7] This hypothesis has been under consideration for more than two years, but has been withheld pending the completion of experiments which show that the chromosomes in question are alive and functional (see below). The possibility of bodies moving through the protoplasm by such means has been suggested to me by several physiologists, particularly Dr. Selig Hecht and Dr. S. O. Mast. To them and to Dr. Ralph S. Lillie I am greatly indebted for advice on numerous questions which have arisen in connection with the present study.

chromosomes do not give the appearance which would be expected if the activity were operating solely on the anterior (distal) ends. This is particularly true in the case of the V-shaped member. Often the two arms of this V are deflected near the ends in opposite directions, yet they do not continue to move in opposite directions and pull the chromosome out into a straight line. Rather, the chromosome as a whole continues to move, even with the apices deflected. This would indicate that the activity is graded along the length of the chromosome—or, perhaps more probably, that it is an activity not only of the stainable portion of the chromosome proper, but also of the chromosome sheath, and hence that it involves a relatively large mass of material whose shape may be modified without seriously affecting the movement.

Any antero-posterior differentiation of the chromosome required by such hypothesis is readily conceivable, since the "insertion point" of the spindle fiber distinguishes the posterior end of the chromosome and may well influence the general activity of this region.

In the above considerations two possibilities have been omitted which may immediately be raised as objections or alternatives to the hypothesis presented. One is the possibility that the movements under consideration result from an electromagnetic activity and simply reflect a repulsion between the four retreating chromosomes and the pole. Perhaps such a possibility cannot be ruled out at present; but the many difficulties it encounters when applied to mitosis in general [8] make its validity seem very doubtful. For this reason it is being considered only as a last resort.

The other possibility is that the chromosome movements under consideration do not represent true mitotic activity at all. Since the four retreating chromosomes are ultimately cast off and degenerate, it may be suggested that they are already inert and degenerating during anaphase and hence that they are to be considered essentially as foreign bodies whose elimination has no connection with the process of mitosis. This possibility seems to be definitely ruled out by the experimental evidence considered below.

If the hypothesis of chromosome movement presented above is correct it should be applicable to ordinary bipolar mitosis. In such ordinary mitosis the two forces or activities here identified because of their antagonistic action would have to act in the same direction and supplement, instead of oppose, one another. This feature will be considered in a subsequent, more detailed, account.

[8] See, *e.g.*, Wilson, 1925, p. 186; Gray, 1931, p. 167.

II. Experimental Modification of the Monocentric Mitosis

As intimated above, when one studies the normal processes of monocentric mitosis in *Sciara* spermatocytes, he is at once confronted by the question as to whether the chromosomes which retreat from the pole are in a normal state of activity or are in a degenerating condition. The answer to this question bears not only upon the immediate problems involved in the present paper, but also upon other aspects of our studies—particularly those concerned with sex determination, the influence of sex on chromosome behavior, and the interrelation between cytoplasm and chromosomes. For this reason especial attention has been devoted to the subject and numerous experiments have been performed in an effort to modify the normal procedure in such a way as to give critical evidence. Such evidence has now been secured. It is considered below, together with other features bearing on the question at issue.

It is evident, as already indicated, that normally the chromosomes which are cast off in the polar-body-like process or "bud" eventually degenerate. This is true not only in the case of the monocentric first spermatocyte division, but also in that of the dicentric second spermatocyte division which, of course, immediately succeeds the first. The ultimate degeneration in both cases is readily understood, because the "bud" contains almost nothing but the chromosomes, and in the absence of cytoplasm these obviously could not long continue their normal development.

The immediate problem is to determine whether, in the monocentric division, this degeneration takes place after the "bud" is extruded or is already well under way during the division itself. Before considering the experimental evidence attention may be called to two features in the normal processes which strongly suggest that the former alternative is correct. The first of these is the fact that after the chromosomes are extruded in the "bud" they appear to undergo changes in form and structure similar to those exhibited by the chromosomes retained in the cell, although it is difficult to trace these activities in detail in the narrow compass of the "bud," and they apparently do not continue through more than the interkinesis period. They do seem to indicate, however, that the discarded chromosomes are not inert at the time they are discarded.

The second line of evidence comes from the second division. It seems clear that, although the chromosomes which are discarded at this division eventually degenerate, they are not degenerating during the mitotic process, for the mitotic figure here is bipolar, the chromosomes show essentially typical behavior, and those which are discarded are sister halves of those which remain in the functional cell (spermatid).

Similar evidence is, of course, provided by the well-known phenomena of polar body formation in eggs, which show that the ultimate fate of chromosomes is not necessarily an indication of their condition during the preceding mitosis.

The final, and apparently conclusive, evidence, however, comes from experiments which show that when retained in the cell, instead of discarded, the chromosomes under consideration (in the monocentric division) remain alive and active, and hence that they must be alive and able to function during the mitosis itself.

The evidence is briefly as follows: Under suitable conditions, such as exposure to low temperatures,[9] the formation of the polar-body-like protuberance may be inhibited at either the first or second divisions. In such cases the chromosomes which would otherwise be eliminated are retained in the cell and are thereby enabled to continue functioning and to give evidence of their condition.

In the monocentric mitosis it is apparently possible to stop the "backward" movement of the retreating chromosomes at any stage of what would correspond to anaphase in typical mitosis. The subsequent positions of these chromosomes, and to some extent their behavior, appears to be determined by the time at which their migration is stopped. This is inferred from the fact that in the preparations examined the chromosomes are in the various positions corresponding to those through which they normally pass during the monocentric mitosis.

The details of these features will be left for a more complete account. Present interest centers in the fact that the retreat of the chromosomes has been stopped at various stages and that preparations have then been made at various subsequent time intervals. In consequence it is possible to reconstruct the sequence of stages showing the behavior of these retained chromosomes during the ordinary transformations of the second spermatocyte.

Development of the second spermatocyte in this treated material apparently progresses in the normal fashion. The chromosomes of the regular nucleus go through the usual interkinesis, prophase, metaphase, and anaphase stages. Coincidently the four artificially retained chromosomes go through a corresponding series of stages up to late prophase or metaphase. They become diffuse or reticular like the others during the telophase and interkinesis (see Fig. 12); they divide, and they condense into split prophase chromosomes during the prophase of the second spermatocyte. They appear to undergo these transformations

[9] The precise nature of the treatment required has not yet been determined. The conditions described here were first found in material purposely subjected to cold. Similar conditions have been observed in a few specimens taken from laboratory stocks which had probably been exposed to low temperature through cooling of the room.

regardless of their position in the cell or the stage at which they were interrupted in the monocentric division. They also appear to act independently of one another, in the sense that they exhibit essentially the same characteristics when widely separated from one another as when close together (see Figs. 13 and 14). When separated each is surrounded by a relatively transparent layer of protoplasm, which I interpret as an integral, although perhaps transitory, part of the chromosome, representing the chromosome "sheath" described by numerous observers in ordinary mitoses.[10] When close together, the chromosomes give the appearance of lying in a small nucleus; but I have been unable to detect a nuclear membrane and am inclined to attribute the appearance to the coalescence or approximation of the transparent layers or sheaths around the chromosomes.

In most cases these artificially retained chromosomes do not exhibit an increase in size corresponding to that of the chromosomes in the normal nucleus. They vary considerably in this respect and the evidence as a whole suggests that the growth is dependent on the position of the chromosome with respect to the normal nucleus—being greatest when there is a close proximity (see Fig. 14). This in turn suggests that the nature of the protoplasm immediately surrounding the chromosome is an essential factor in respect to growth, and that only the environment provided by the nucleus itself is suitable for promoting normal growth.

In no case has it thus far been possible to inhibit the monocentric mitosis in such a way as to retain all the chromosomes in the nucleus and thus get a second spermatocyte nucleus containing the total diploid group; but it seems almost certain from the above evidence that if this could be done the growth and activity of the chromosomes which are ordinarily eliminated (paternal members) would be equivalent to that of their (maternal) homologs.

As would be expected, the correspondence between the transformations of the retained chromosomes and the others ceases at the metaphase of the second spermatocyte division. In the absence of any mitotic apparatus (asters or spindle) the daughter halves of the artificially retained chromosomes cannot separate, hence they remain together during the anaphase of the second division. Whether or not they would subsequently exhibit further activity during the development of the spermatid has not been determined.

From the evidence presented above it seems clear that in the monocentric mitosis under consideration the retreating chromosomes are alive and are capable of taking an active part in the mitotic movements as postulated in the first part of the paper. This does not, of course, mean that they are necessarily entirely unaffected by the monocentric mitotic

[10] A good example is described by Metz and Nonidez, 1924.

phenomena under normal conditions, or that their activity is precisely like that of their homologs which go toward the pole. Indeed, it seems evident that a difference in activity does exist, that it is due to the sex of the parent from which the chromosomes were derived, and that it is in some way responsible for the opposite responses of the two sets of chromosomes.

Summary

I

The present paper supplements previous studies on the monocentric first spermatocyte division in *Sciara* and considers the findings in relation to the general problem of the mechanism of mitosis. Contrary to generally accepted principles of mitosis, an accurate segregation of chromosomes is effected in a unipolar field during the mitosis in question. The segregation is highly selective in that paternal chromosomes react in opposite fashion from their maternal homologs and pass away from the pole instead of toward it. These chromosomes which "retreat" from the poles exhibit characteristics which are believed (1) to demonstrate the functional reality of the so-called "spindle fibers," which in this case retard the movement of the chromosomes, and (2) to suggest that the opposing force or activity responsible for the movement of the chromosomes is due to an activity of the chromosomes themselves. The hypothesis of "autonomous" movement on the part of the chromosomes is suggested particularly by two lines of evidence:

(1). Each retreating chromosome in its movement directly away from the pole acts independently of the others and may move along any radial line within the theoretical cone delimited by imaginary extension of the half spindle. Its path appears to be determined entirely by its original position with respect to the pole.

(2). Each retreating chromosome appears to move in a small mass of protoplasm derived from the original half spindle, and to maintain its position at the *apex* of this mass during the movement. On account of the opposing activity represented by the so-called "spindle fiber," it seems improbable that the chromosome could retain this apical position if it were not itself responsible for the movement.

The suggestion is made that the anaphase movement of chromosomes may be primarily due to such activity of the chromosomes themselves and that this activity serves to bring about movement by producing localized alterations in the viscosity of the adjacent protoplasm.

II

Experimental evidence is reviewed which shows that the retreating chromosomes are alive and therefore theoretically capable of function-

ing as postulated during the movements in question. This evidence also bears on the interrelations between sex and chromosome behavior to be considered subsequently.

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PLATE I

All figures are from spermatocytes of *Sciara coprophila* Lintner, fixed in Gilson's mercuric-nitric fluid and stained in Heidenhain's iron haematoxylin. Figures 7–14 are from camera lucida drawings made at table level, using 1.5 mm. objective and No. 12 ocular. They are reduced approximately three-fifths in reproduction. The drawings were made by Dr. Esther Carpenter, with the exception of that for Fig. 11 which was made by Miss Louise H. Buck. Figures 7–10 are from un-

treated specimens showing certain features of the normal monocentric mitosis; Fig. 11 from a specimen treated with cold; Figs. 12–14 second spermatocytes from specimens presumably exposed to cold in the laboratory during the first division. Figures 7–10 are from sections; 11–14 from smears.

FIG. 7. Primary spermatocyte in approximately the stage represented in Text Fig. 4. The group of six chromosomes has reached the pole and lost its earlier orientation. The four retreating chromosomes have begun to converge after reaching the periphery. Note the two chondriosomes separating the V-shaped chromosome at the left from the others and showing that this chromosome has moved through part of the chondriosome mass (only part of which is shown).

FIG. 8. Slightly later stage, corresponding approximately to that shown in Text Fig. 5. The chromosome in black at the right has apparently passed through the mass of chondriosomes along the path indicated. The chondriosomes in the center lie at about the level of the chromosome at the extreme left, which may have passed through the opening between the two clusters.

FIG. 9. Slightly earlier stage than the preceding. In this cell the four retreating chromosomes are not widely separated. They have come into contact with the saucer-shaped mass of chondriosomes preparatory to passing through it. The contact has evidently served to bend or fold their distal ends, giving a clumped appearance. This is shown clearly by the V-shaped chromosome at the left. Although not very evident in the figure, the transparent sheath around each chromosome is distinct in the preparation; it serves to keep the chromosome proper from touching the chondriosomes.

FIG. 10. Incomplete figure at approximately the same stage as the preceding. Three retreating chromosomes are shown—two rods and the V. The one at the left is at a higher level than the others and is completely cut off from the periphery by chondriosomes, only a few of which are shown. The sheath surrounding this chromosome is in contact with the chondriosomes. This chromosome would subsequently have passed between the chondriosomes to the periphery. Such cases seem to demonstrate that the chromosomes are not transported by currents and that each is at the apex of the outflowing mass of protoplasm in which it lies, if there is any such mass in addition to the transparent "sheath."

FIG. 11. Primary spermatocyte from smear made from material treated with cold for 48 hours and dissected and fixed in cold. Cell slightly flattened. The four retreating chromosomes are long and slender and widely divergent—the V-shaped one lying at the upper left of the figure. Whether or not their elongation is due to the cold is uncertain, but it is probable that their movements were greatly slowed down, if not stopped, by the treatment. The chromosome at the lower right lies partly among the chondriosomes and is curved partially around one of them as shown in the figure. The achromatic figure is not visible in this cell.

FIGS. 12–14. Second spermatocytes in which the chromosomes which should have been discarded at the first division have been retained, presumably due to exposure to cold.

FIG. 12. Interkinesis stage showing the chromosomes of the regular nucleus above and the artificially retained ones in a cluster resembling a nucleus below. The latter chromosomes are exhibiting the same interkinetic changes as those in the regular nucleus.

FIG. 13. Later stage from same testis as the preceding, showing the regular chromosomes, above, in metaphase of second division, seen in side view (compare with figures in Metz, Moses, and Hoppe, 1926) and the artificially retained chromosomes near lower right. The latter have divided and condensed and are in essentially the same stage as the others. Each is surrounded by its transparent "sheath," which in this case is clearly revealed by the relatively dark cytoplasm surrounding them.

FIG. 14. Later stage (middle anaphase) of second division from same testis. As in preceding three cases (all from smears) the achromatic part of the mitotic figure is practically invisible (see above under "methods"). The position of the spindle is clearly indicated, however, by the orientation of the chromosomes passing to the poles (for explanation of this mitosis see Metz, Moses, and Hoppe, 1926). The four lowermost chromosomes, all split, are the artificially retained ones—marked *r*. They lie near, but not in, one end of the spindle. The one shown in solid black lies at a high level directly above this pole of the spindle. Note that in this cell the retained chromosomes appear to be fully as large as those from the regular nucleus. In the cells of this testis only one large "limited" chromosome is present—a condition frequently found. The daughter halves of this chromosome in the present figure lie at the left of the spindle, going to opposite poles. The daughter halves of the small V are at the right of the spindle.

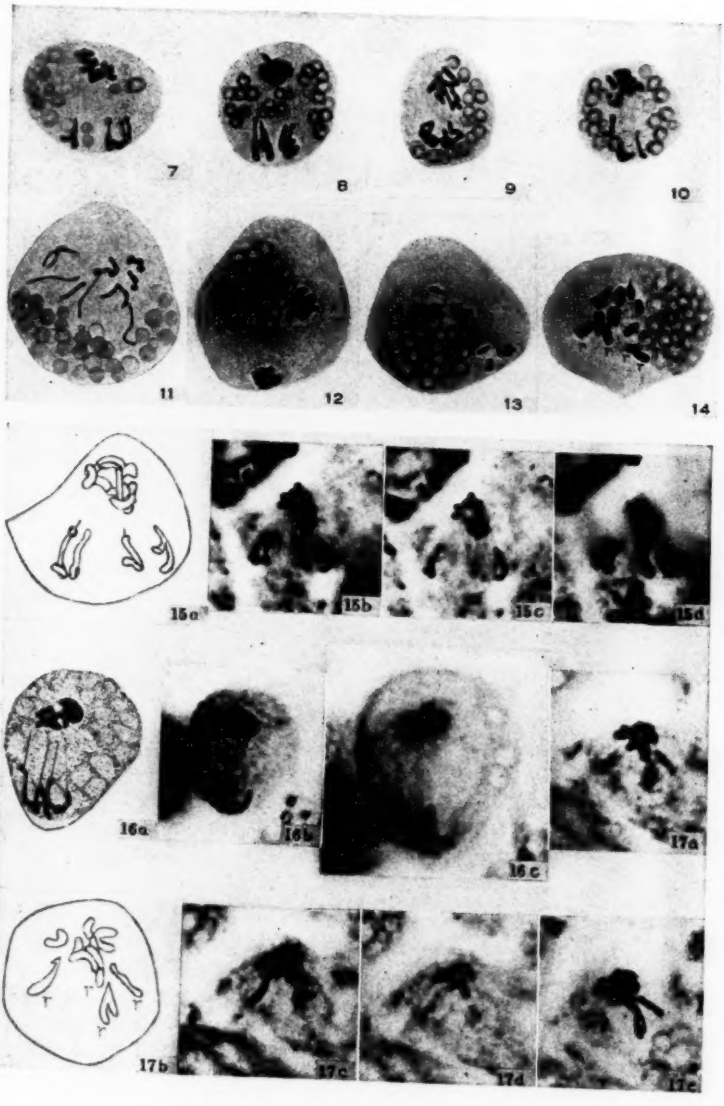
FIGS. 15–17. Photographs with explanatory drawings of three primary spermatocytes during the monocentric mitosis; from sections.

FIG. 15. A cell showing the retreating chromosomes shortly after they have reached the periphery and their paths have begun to converge. The chromosomes at the pole have lost their orientation and become irregularly clumped. *a*. Outline camera drawing; note that the V-shaped chromosome is at the right. *b*. Composite photograph taken at different levels to show relative horizontal positions of the retreating chromosomes. The two outer ones are at a high level and the two inner ones at a low level. *c* and *d*. Photographs at high focus and low focus, respectively, showing the features just mentioned.

FIG. 16. Similar illustrations of a cell at a slightly later stage. The V-shaped chromosome, near middle of the retreating group, and the rod at the right are at higher levels than the other two. *a*. Camera drawing (see Fig. 17, Metz, Moses, and Hoppe, 1926). *b*. Photograph at high focus showing the two upper chromosomes, with indications of the other two which are not entirely out of focus. *c*. Photograph at low focus, using higher magnification, showing the two lower chromosomes. As indicated by the figures, the paths of the retreating chromosomes are converging. The two lower members are moving upward and toward the right; the V is moving downward, and the rod at the right is moving downward and toward the left.

FIG. 17. Similar illustrations of an unusually small cell at an earlier stage than either of the two preceding. Three of the retreating chromosomes, two rods and the V, are readily seen at short distances from the other chromosomes, which are close together. *a*. Composite photograph taken at different levels. *b*. Outline camera drawing designed to supplement the composite photograph in showing the general composition of the figure, and the comparative horizontal positions of the chromosomes. Apparently the fourth retreating chromosome is the long one projecting from the compact group down toward the retreating V. This member is not completely separate from the group which has gone to the pole, although it lies below most of the members of this group. The identity of this chromosome is not certain, however, because the cell may be cut, and also the clustered chromosomes are too closely clumped to make individual identification possible. Note the wide divergence of the three conspicuous retreating chromosomes. Presumably none of them has quite reached the periphery. *c*. High focus showing the retreating hooked rod, at left. *d*. Lower focus showing the group near the pole and also indicating the vertical distance between the retreating rod just mentioned and the two other members shown in the next figure. *e*. Still lower level showing the retreating V and the rod seen at the right in *a* and *b*.

PLATE I



CHANGES IN SUSCEPTIBILITY OF DROSOPHILA EGGS TO ALPHA PARTICLES

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In a previous report (Henshaw and Henshaw, 1933) we have shown that *Drosophila melanogaster* eggs vary markedly in susceptibility to 200 kv. X-rays at different stages during early development. A correlation of the changes in radiosensitivity with the changes in development showed that they become more sensitive during cleavage and blastulation, much more resistant at or near the beginning of gastrulation, and more sensitive again as gastrulation gets under way. Having found that susceptibility to one type of radiation varies in a certain way, it is of interest to determine whether it varies in the same way when other types of radiation are used.

Alpha particles, beta particles, and gamma rays are the three recognized forms of radiation emitted by radioactive substances. The alpha and beta rays are corpuscular in nature while the gamma radiation is like X-rays of very short wave length. All of these forms of radiation, including X-rays, are capable of causing ionization in matter subjected to their influence. According to the Rutherford-Bohr theory of the structure of the atom, the alpha particle is identical with the helium atom stripped of its two planetary electrons. It therefore has an electric charge of two positive and a mass about 7000 times that of the beta particle or electron (Failla, 1927). Since it has atomic dimensions and has a velocity of .15 to .20 times that of light, it represents an enormous concentration of kinetic energy. "In fact the alpha particle is the most potent agent known to science" (Lind, 1928).

In addition to the 200 kv. X-rays used previously, we have had at our disposal 40 kv. X-rays, gamma rays, and alpha particles. Tests determining susceptibility changes have been made with each of these. The responses to 40 kv. X-rays and gamma rays were essentially the same as to 200 kv. X-rays. With alpha particles, however, the effects were very different. Experiments with alpha particles will therefore be presented in detail, after which results for all will be compared.

Alpha Radiation

The source of alpha particles was polonium, which is one of the radioactive products of radium. Polonium was extracted from the walls of old radon tubes which had been used in cancer therapy. This was done with a dilute acid solution (.2 per cent HCl, approximately). The extract solution was flooded onto one surface of a copper disc 3.5 cm. in diameter and the polonium deposited on the metal surface. The amount retained by the disc was sufficient to kill *Drosophila* eggs after several minutes exposure. That alpha particles were responsible for the killing observed and not some other form of radiation was made certain by experiments which will be described later.

Treatment of Eggs

A large collection of eggs was obtained from actively laying flies during a two-hour period.[1] This was divided into several portions, usually fifteen, each containing more than 100 eggs and often more than 200. With a soft brush the eggs were spread evenly in a single layer on small pieces of filter paper and were then ready for treatment.

The exposures were made in moist chambers, arranged by placing moist filter paper on the bottom of Petri dishes 15 cm. in diameter. The filter paper strips carrying the eggs were placed on these near the center. The copper disc was fastened in the center of the Petri dish cover so that the plated surface was directed toward the eggs and was about two centimeters above them when the dish was covered in the usual way. Eggs placed directly under the disc were killed by the radiation while those placed 5 cm. or more away were unaffected.

In order to determine the susceptibility of the material at different ages, tests were performed on different parts of the same collection of eggs at hourly intervals. The first began 15 minutes after the end of the collection period. Four samples were used for each test; the first three received 15, 30, and 45 minutes exposure, respectively; the fourth was kept as a control. Three exposures were used in order that the effect of different amounts of radiation could be considered. After treatment the samples and the control were put away in moist chambers at room temperature (22–25° C.). On the third day after irradiation, counts were made to determine the percentage hatched. This served as the criterion of effect.

Results

The data obtained are shown in the accompanying table. Since age in development is the point in question, age is given relative to the time

[1] For culture of flies and the collection of eggs, see the earlier report—Henshaw and Henshaw.

of fertilization rather than the time of collection. Since the eggs are fertilized individually as they pass through the vagina of the female fly and are deposited at any time during the collection period, their age is

TABLE I

Data on Changes in Susceptibility of Drosophila Eggs to Alpha Particles

	Exp. No	Age in Hours							
		1½	2½	3½	4½	5½	6½	7½	8½
		Percentage Eggs Hatching							
Controls	1	86	93	96	94	87			
	2	91	92	88	89				
	3	90	94	96	91	95		93	
	4	97	93	91	96	92	93		93
	5	93	99	93	89	98			
	6	98	97	99	99	98			
Average		92	93	94	93	94	93	93	93
15 min. exposure	1	90	42	11	37	74			
	2	84	35	5	30				
	3	88	42	33	41	50		82	
	4	88	23	13	34	52	74		
	5	89	72	28	66	89			
	6	83	21	4	19	49			
Average		87	39	16	38	52	74	82	
30 min. exposure	1	81	22	5	15	32			
	2	81	22	4	10				
	3	85	18	20	22	19		83	
	4	71	10	5	8	28	44		81
	5	46	35	29	22	84			
	6	71	10	6	6	17			
Average		76	19	11	14	36	44	83	81
45 min. exposure	1	86	1	3	11	21			
	2	71	9	1	4				
	3	68	11	18	19	14		60	
	4	56	12	11	5	9	35		61
	5	49	23	26	18	79			
	6	45	8	10	6	29			
Average		63	11	11	11	30	35	60	61

known to vary as much as two hours. It is necessary, therefore, to speak of an average age for the eggs in a sample. As pointed out in the

earlier report, this is approximately the middle of the laying period. Thus if age is reckoned from the average time of fertilization, the eggs are one hour old at the end of collection.

As seen from Table I, a control sample was kept for every age group irradiated. Among these the fertility varied from 87 to 98 per cent. The average, therefore, was 92 per cent; this was taken as 100 per cent and other values were adjusted accordingly. The average values were thus treated and plotted in Fig. 1 (solid line curves).

The abscissa (Fig. 1) shows age in hours and stage in development, while the ordinate shows the percentage of eggs hatching. The curves, therefore, show the percentage of eggs hatching at different ages after receiving 15, 30, and 45 minutes exposure to alpha particles. It will be seen that the eggs become increasingly sensitive during the first two or three hours after fertilization, after which time they become more resistant again. The point of maximum sensitivity (minimum resistance), therefore, is reached between 2 and 4 hours after fertilization.

The dash line curves show data obtained previously for 200 kv. X-rays. The shape and position of such curves vary depending upon the dosage of radiation administered. Accordingly, those for 2.5 (*A*) and 5 (*B*) minutes exposure (234 rœntgens per minute) were selected to show the most prominent changes. In the previous experiment a series of different dosages were used, but without exception there was a distinct increase in resistance between 2 and 4 hours after fertilization. The subsequent fall in resistance shown by curve *B* appeared only when larger dosages were given. By comparing directly the curves for the two types of radiation it is seen that, in general, they vary just opposite. While the eggs become more resistant to 200 kv. X-rays, they become more susceptible to alpha particles; the point of greatest resistance to 200 kv. X-rays and the point of greatest susceptibility to alpha particles being reached at about the same time.

Less complete data for 40 kv. X-rays (soft X-rays) and gamma rays are shown in Fig. 2. Only one dosage was used in each case. The curves for these radiations are similar to the one for 2.5 minutes exposure to 200 kv. X-rays. Presumably, therefore, if dosages had been varied for these radiations as for 200 kv. X-rays, similar variations due to dosage would have been observed. Whether this is actually the case can be determined only by further experiment, but the evidence is sufficient to show clearly that the organisms become more resistant to 40 kv. X-rays, 200 kv. X-rays, and gamma rays when they become more susceptible to alpha particles.

The problem, therefore, is to account for the difference in response to alpha particles. Since the response varies for different radiations

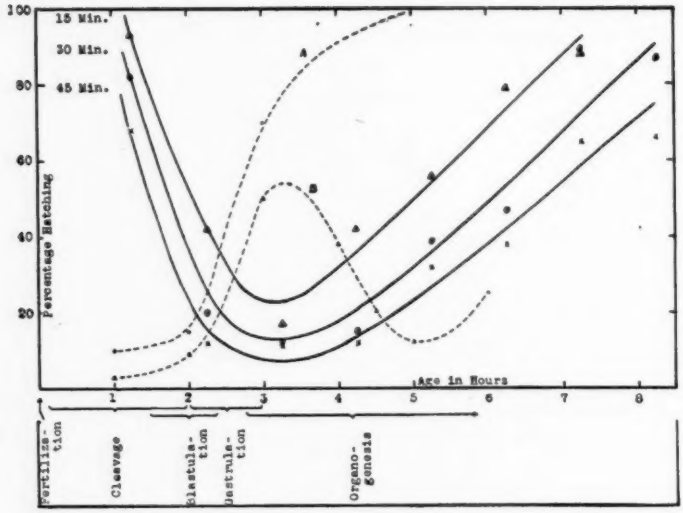

FIG. 1

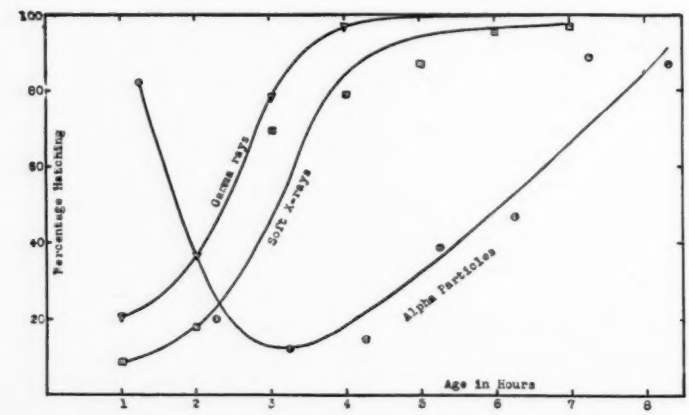

FIG. 2

FIG. 1. Curves showing changes in susceptibility to alpha particles (solid line curves) and 200 kv. X-rays (dash line curves). The index at the base of the figure shows the stage in development at different ages.

FIG. 2. Curves showing the changes in susceptibility to 40 kv. X-rays (soft X-rays), gamma rays, and alpha particles.

and different ages, it is evident that both the character of the radiation and the processes of development are involved. These will be discussed in turn.

Properties of the Radiations

One of the essential differences between the radiations used is the power of penetration into matter. "As a rough working rule, it may be taken that beta particles are about 100 times as penetrating as alpha particles and that gamma rays are from 10 to 100 times as penetrating as the beta rays. . . . A thickness of .006 cm. of aluminum or mica or a sheet of ordinary writing paper is sufficient to absorb completely all the alpha particles" (Rutherford, Chadwick, and Ellis, 1930). Since, therefore, the large supply of kinetic energy possessed by the alpha particles is expended in so short a distance, much greater destruction is caused per unit length of their trajectory.

Alpha particles of polonium are completely stopped by 4 cm. of air, while beta particles require 5 mm. of aluminum or 1 mm. of lead to stop all (Lind, 1928). Gamma rays, on the other hand, will pass through 15 cm. of lead and the softer X-rays will pass through 1 mm. or more of aluminum. It is certain, therefore, that gamma rays and X-rays and some beta particles are capable of penetrating to all parts of the *Drosophila* egg. Unfortunately, microscopic preparations of eggs which have been exposed to alpha particles do not show delimited changes which mark the extent of penetration.

Alpha particles from polonium penetrate water a distance of 32 μ (Michl, 1914). Presumably, they penetrate about the same distance into protoplasm, since it has approximately the same density. Feichtinger (1931) reports that alpha particles of polonium produce visible effects in root tips to a depth of 30 μ. Zirkle (1932) described the effects of this radiation on the spores of the fern, *Pteris longifolia,* which have a diameter of about 38μ. The nucleus in this form ordinarily lies to one side of the center. When irradiated with the nucleus directed away from the source of radiation the effect was very different from that obtained when it was on the side directed toward the source. In this case, the particles must have penetrated to about half the diameter of the organism or approximately 20 μ. While carrying out some work on eggs of the sea urchin, *Arbacia punctulata,* we also have obtained some idea of the penetration of alpha particles into protoplasm.

Arbacia eggs, when placed in a hanging drop, settle to the lower surface where they can be irradiated with alpha particles. A sample of eggs exposed for 10–15 minutes while in the two-cell stage and examined at subsequent intervals will contain deformed individuals similar to those shown in Fig. 3, row *A*. If exposed while in the four- or eight-

cell stages, figures like those in rows *B* and *C*, respectively, will be found. Normally, the cells divide equally and simultaneously during early cleavage so that the number of cells increases 2–4–8–16, etc. It appears that during exposure some of the eggs by random distribution took a position with one cell (in the first case) shielded by the other from the radiation and that the radiation penetrated only far enough to destroy the cell nearest the surface. Since the over-all diameter of this organism in the two-cell stage is about 75 μ and since alpha particles appear to have penetrated not more than half this distance, their range in this case must have been less than 40 μ.

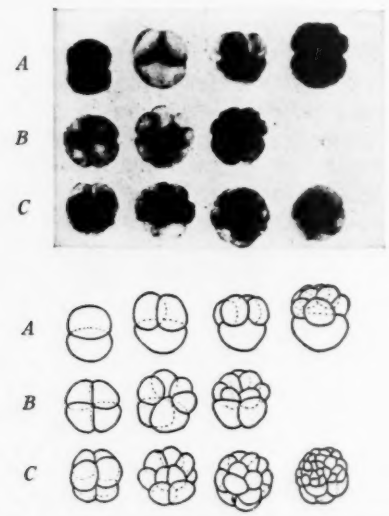

FIG. 3. Photomicrographs and diagrams showing the effects of alpha particles on *Arbacia* eggs when exposed in the 2-cell (*A*), 4-cell (*B*), and 8-cell (*C*) stages.

From the instances cited, it seems clear that the range of alpha particles in protoplasm is approximately 30 μ. Since then, the transverse diameter of the *Drosophila* egg is about 100 μ, it is improbable that the particles ever reach their center. In the case of the experiment above they were probably stopped near the surface since they passed through 2 cm. of air and the membranous chorion before reaching the living parts. Since the difference in results obtained with the different radiations may be accounted for on the basis of penetration (see below), other properties of the radiations need not be considered.

The tests performed to make certain that alpha particles and not some other radiation were responsible for the results obtained depended on differences in penetration range of the different radiations and may be given briefly at this point. An ordinary piece of writing paper was placed over the polonium-plated disc during exposure in one case, and the eggs were placed at a distance of 4 cm. from the disc in another. In both cases no eggs were killed, although the time of exposure was continuous from the end of collection to the time of hatching. Since the

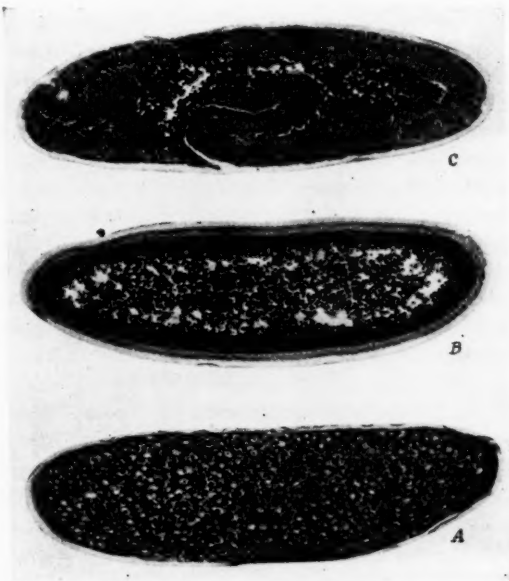

FIG. 4. Longitudinal sections of *Drosophila* eggs of different ages: (*A*) before nuclei begin to migrate to periphery, 0–1.5 hours old after fertilization (*B*) blastular stage, 1.5–2.5 hours after fertilization; and (*C*) gastrular stage, 2–3 hours after fertilization.

beta and gamma rays which might be present would have a greater range than 4 cm. of air and would not be stopped by a sheet of paper, the observed effects may safely be attributed to alpha particles.

EARLY EMBRYOLOGY IN DROSOPHILA

Development as it pertains to the present discussion may be described briefly. The egg and sperm nuclei unite near the center of the egg and the early cleavages, nuclear only, take place synchronously in

the central region at the rate of 1 in 10–12 minutes at room temperature (22–25° C.). At about the eighth or ninth cleavage (Huettner, 1923), approximately 100 minutes after fertilization, the nuclei begin to migrate to the periphery where cell membranes are formed around them and where they arrange themselves in a single layer to form the blastoderm. This thickens by continued mitosis and growth and at about 150 minutes after fertilization gastrulation begins by invagination (Figs. 1 and 4).

Correlations

By correlating the changes in sensitivity with the location of the cells at different stages, it is seen that the eggs become increasingly susceptible as the nuclei move toward the periphery, that they are the most susceptible when the largest number of cells are active at the periphery, and that they become more resistant as the actively growing regions move away from the surface toward the center again. Because of this it is believed that the changes in susceptibility to alpha particles may be accounted for by saying that (1) the amount of radiation used is sufficient to produce death only when active cells (or nuclei) are affected; that (2) at first, the cells, being in the central region of the egg, are beyond the range of the particles; that (3) they move into the range as they pass to the periphery; and that (4) the active cells move out of the range again at the time of gastrulation.

Summary

1. Changes in the susceptibility of *Drosophila* eggs to different kinds of radiation have been followed during early development.

2. It was found that the eggs become more susceptible to alpha particles during cleavage and blastulation and more resistant during organogenesis, the point of maximum sensitivity being reached at or just after the beginning of gastrulation.

3. A comparison of these findings with those obtained for 200 kv. X-rays showed that the eggs were most resistant to the X-rays when they were most susceptible to the alpha particles.

4. Further experiments with 40 kv. X-rays and gamma rays indicated that the organisms were consistent in their responses to radiation capable of penetrating uniformly to all parts.

5. It appears, therefore, that the difference in response to alpha particles was due mainly to two factors: (*a*) the short penetration range of the radiation into protoplasm, and (*b*) the movement of the active cells into and out of its range.

Acknowledgments: The writers wish to express their indebtedness to Dr. G. Failla for suggesting the use of alpha particles and for his aid and helpful suggestions during the course of the work.

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ACTION OF ELECTROLYTES ON THE DORSAL MEDIAN NERVE CORD OF THE LIMULUS HEART

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The neurogenic origin of the heart beat of *Limulus* has been demonstrated by Carlson (1909) and confirmed by the more recent work of Garrey (1930) and Samojloff (1930). This demonstration depends upon the fact that the dorsal median nerve cord, where the rhythm originates, can be separated almost entirely from the heart musculature without interrupting their connection, so that experiments can be performed on the nerve and on the musculature separately. Although the literature on the effect of electrolytes on the vertebrate heart and on certain types of invertebrate heart has been very extensive since Ringer's time, relatively few papers have appeared (chiefly by Carlson himself) on the actions of salts on the *Limulus* heart. It seemed, therefore, highly desirable that further work of this kind should be carried out, especially in reference to the action of salts on the automatic nervous rhythm.

The present work was done on the nerve only. The dorsal median nerve cord (or ganglion) was isolated from the posterior end of the heart, remaining attached anteriorly, and the heart was sectioned after the second segment. This preparation, first used by Carlson (1906), is similar to a nerve-muscle preparation. The regular contraction of the heart depends upon the rhythmic nervous discharge, originating in the ganglion; and the amplitude of contraction depends upon the intensity of the nerve impulse; presumably this depends upon the number of nerve cells acting together. The first two segments of the heart were mounted in Ringer's solution and attached to a light heart lever for graphic registration with the nerve hanging down into the test solution. Any such solution could be quickly changed for another without disturbing the preparation.

Instead of using sea water as an indifferent medium for the ganglion, a simplified medium similar in composition to Ringer's solution and isotonic with the Woods Hole sea water ($\Delta = 1°.82$) was used. This modified Ringer's solution consisted of 100 vols 0.52 M NaCl, 2 vols. 0.52 M KCl, and 15 vols. 0.29 M $CaCl_2$, and proved highly satis-

factory as a substitute for normal sea water or *Limulus* plasma. In this medium both the nerve and the whole heart could be kept in good condition for several hours, although in some cases there was a slight temporary increase in rate during the first few minutes after transfer from sea water to the medium. The hydrogen-ion concentration of this solution was slightly on the acid side of neutrality (pH ca. 6); the same was true of the other salt solutions used in this work. Any effect due to the change of hydrogen-ion concentration was inappreciable in these experiments, except in a few cases to be described later.

Isotonic solutions were used throughout. When the salt concentration, considered by itself, was lower than isotonic, the solution as a whole was made isotonic by the addition of sucrose. According to Garrey's determinations (1915), 0.52 M NaCl, 0.29 M $MgCl_2$, and 0.73 M sucrose are isotonic with the Woods Hole sea water, and hence with the Ringer's solution and the blood. All experiments were done at room temperature; this varied from 21° to 27° C. The action of a given salt solution was indicated by the change of activity observed in the heart muscle when the Ringer's solution surrounding the ganglion was exchanged for the experimental solution at the same temperature. All solutions were in gaseous equilibrium with the air and uniform in pH.

The Action of Single Salts

Neutral Sodium Salts

As shown by Carlson (1906*a*), the first effect of pure isotonic NaCl solution is to increase the rate of the nervous rhythm; this effect is followed by decrease in the intensity of the nervous discharge, as shown by lessened height of contraction, irregularity of rhythm, and ultimate standstill. The time for the abolition of the rhythm varies from half an hour to an hour or more. The height of contraction may increase somewhat at the beginning or may not. The invariable effects are (1) the rapid rate, (2) the irregularity of the rhythm and amplitude and (3) the gradual decrease and final cessation of contractions. All the other neutral sodium salt solutions have similar actions, differing only in degree. The quantitative differences between the neutral sodium salts follow the anion series of Hofmeister (lyotropic series); this can be demonstrated in several ways, as follows:

(*a*) By the relative effectiveness in increasing the rate of the nervous rhythm. When a nerve is transferred from Ringer's solution to a solution of pure NaCNS, for instance, the rate (contractions per minute) is only slightly increased, but the height of contraction falls off rapidly and the rhythm ceases in a minute or two. NaI has exactly the same action, except that the rate of increase is greater, and the rhythm

is maintained longer, usually for two to three minutes. A specific toxic or inhibitory effect of the pure Na-salts is thus shown, for when all electrolytes are removed by surrounding the ganglion with isotonic sucrose solution, the rhythm persists for some time, frequently for half an hour or more. The relative effectiveness of the different salts in increasing the rate shows the order of the Hofmeister series, NaCNS being the least and Na_2SO_4 the most effective. Table I and Fig. 1 show the typical effect on the rhythm in the different solutions during the first five minutes after transfer from Ringer's solution, expressed as percentages of the original rates in Ringer's solution.

The data in Table I were obtained with a single heart. The ganglion was first treated with NaCNS solution until the rhythm ceased com-

TABLE I

Relative effectiveness of the neutral sodium salts in increasing the rate of rhythm. Numbers represent percentage increases in rate over the control, *i.e.*:

$$\left(\frac{\text{rate in test solution} - \text{rate in Ringer}}{\text{rate in Ringer}} \times 100\right)$$

	1st min.	2d min.	3d min.	4th min.	5th min.
NaCNS	18	stopped			
NaI	72	77	stopped		
NaBr	54	84	124	147	155
$NaNO_3$	82	145	165	170	160
NaCl	96	129	170	205	235
Na_2SO_4	210	305	480		

pletely; the NaCNS was then replaced by Ringer's solution; rhythm, at first slow, was resumed, becoming normal in rate in a few minutes. The Ringer's solution was then replaced by the second solution in the series (NaI), and the beats were counted as before until cessation. Rhythm was again restored by return to Ringer's solution as before, and the next salt solution was used similarly, and so on in the order indicated in Table I. The series was then repeated with the same solutions in the reverse order; Ringer's solution being first replaced by Na_2SO_4, then by NaCl and so on. The effect of treatment for five minutes with the Na-salt solution was always perfectly reversible; in every case normal rhythm returned after transfer to Ringer's solution. The ganglion was apparently uninjured at the end of the series. Each figure in Table I thus represents the average of two determinations on the same ganglion.

The relative effectiveness of the different salts in accelerating the rhythm shows the order:

$$Na_2SO_4 > NaCl > NaNO_3 > NaBr > NaI > NaCNS.$$

It is remarkable that, although NaCNS is the least effective in accelerating the rhythm, it is the most effective in causing standstill; *i.e.*, the order of relative toxic action of these salts is the reverse of the order of effectiveness in increasing the rhythm (with the apparent exception of Na_2SO_4, which differs from the others in being a calcium-precipitating salt). The same order of relative toxicity is also found with ciliary movement, another form of automatic rhythmic activity (Lillie, 1906, 1909; Höber, 1909), with spermatozoa and eggs (Gellhorn, 1927 *a* and

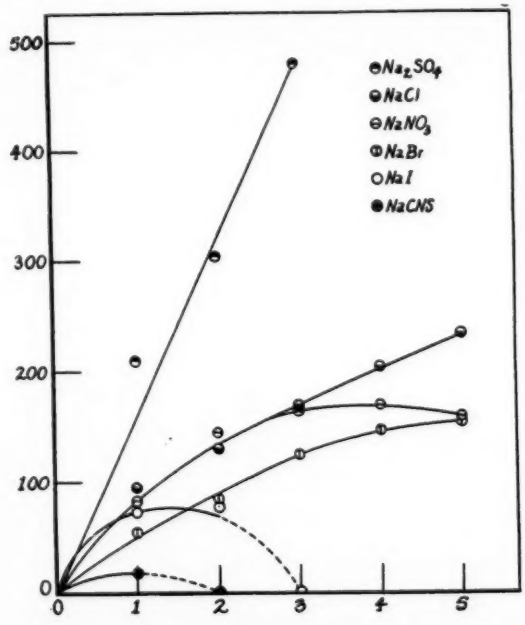


FIG. 1. Relative effectiveness of the neutral sodium salts in increasing the rate. Ordinate: percentage of increase in rate. Abscissa: time in minutes.

b), and with many other cells and tissues (Höber, 1926). In general, therefore, the order of toxicity of the series of Na-salts on *Limulus* nerve agrees very well with that observed in other tissues, as well as with the order of relative action on colloidal systems.

(*b*) The same order of relative toxicity is also shown when the NaCl in Ringer's solution is replaced by equivalent amounts of the other Na-salts. In such a modified Ringer's solution the ganglion shows a decline in activity, which is well marked at the end of ten minutes or sooner

and varies with the nature of the substituting salt. In Table II the effects observed in a typical series are summarized.

All the Na-salts, except Na_2SO_4, on replacing NaCl in Ringer's solution, decrease both rate and amplitude, and the order of relative effectiveness again follows the Hofmeister series. Na_2SO_4 alone increases the rate, apparently because of its calcium-precipitating property. Calcium sulphate was actually seen to be precipitated in the solution. This decrease in calcium would naturally remove part of its antagonistic effect and increase the rate (see below).

In the frog's heart the replacement of NaCl in Ringer's solution by other Na-salts gives a similar series, but the order of relative action is reversed. While Na-salts of CNS, I, Br, and NO_3 increase the amplitude, frequency, and pulse volume, Na_2SO_4 decreases them (Handovsky, 1923) (Sakai, 1914). On the theory that the quantitative differences in the physiological effects of the Na-salts are to be referred to their differences of action on the colloidal structures of the living tissue (Höber, 1926), it would appear that the colloidal structures directly affected by the salts differ in the *Limulus* nerve and in the vertebrate heart in such a way that the orders of relative action are opposed. Differences of this kind are not uncommon in living tissues (see below).

TABLE II

Changes in rate and amplitude on replacing NaCl in Ringer's solution by other Na-salts. Numbers give the percentage change of rate for 10 minutes as compared with normal rate in Ringer's solution.

	Percentage change of rate	Amplitude
Na_2SO_4	+30	gradually decreasing, about 2/3 normal at end of 10 minutes
NaBr	−18	slight decrease but regular
$NaNO_3$	−34	more decrease and less regular
NaI	−37	rapid decrease and irregular
NaCNS	−46	decrease more rapid and more irregular

(*c*) The same series is also shown in *Limulus* nerve when we compare the relative effectiveness of the salts in initiating new rhythm after immersing the ganglion for varying lengths of time in solutions of $CaCl_2$, KCl, non-electrolyte, or in other unbalanced media which abolish the rhythm. To produce this effect isotonic $CaCl_2$ was found to be the most convenient solution, since this salt is less toxic than KCl and is

more rapid in its effect than a non-electrolyte, such as sugar. When a nerve is transferred from Ringer's solution to pure isotonic $CaCl_2$, the rhythm ceases in about one minute. If the nerve be then transferred to a Na-salt solution, new rhythm is initiated sooner or later, the interval depending upon the time of immersion in $CaCl_2$ and the nature of the Na-salt. The results of a typical series of such experiments are seen in Table III, in which are given the intervals required for the reappearance of the rhythm in the isotonic solutions of the various Na-salts after inhibiting the ganglion by immersion in isotonic $CaCl_2$ for one to three minutes.

The relative effectiveness in initiating new rhythm follows the order

$$Na_2SO_4 > NaCl > NaNO_3 > NaBr > NaI > NaCNS.$$

TABLE III

Intervals after which new rhythm appears in isotonic solutions of neutral Na-salts after inhibition by immersion in isotonic $CaCl_2$ for varying lengths of time.

	Duration of $CaCl_2$ treatment		
	1 minute	2 minutes	3 minutes
sea water			immediate recovery
Na_2SO_4			less than 50 sec.
NaCl		less than 30 sec.	after 55 sec.
$NaNO_3$		after 35 sec.	after 2 min. and 15 sec.
NaBr		end of 4th min.	after 3 min.
NaI	35–60 sec.		no recovery in 5 min.
NaCNS	end of 5th min.		no recovery in 5 min.

A balanced solution like sea water is more effective than any pure Na-salt solution.[1]

(*d*) It was shown by Schwarz (1907) and later by Gellhorn (1932, *a* and *b*) that if a frog's skeletal muscle be fatigued to loss of irritability by repeated stimulation while immersed in a solution of a given Na-salt (diluted with isotonic sucrose or Ringer's solution), it will recover irritability when transferred to pure solutions of certain other salts but not in all, the possibility of recovery depending upon the position of the salt in the Hofmeister series. If, for example, a muscle be fatigued in

[1] For restoring the rhythm in the frog's heart after standstill in isotonic sugar solution, Gellhorn (1932*a*) found NaCNS to be the most effective salt, the other Na-salts showing the lyotropic order of relative effectiveness but in the reverse order to that found in *Limulus* nerve.

$NaNO_3$, it will recover in a solution of any salt on the right of $NaNO_3$ in the series

$$Na_2SO_4,\ NaCl,\ NaNO_3,\ NaBr,\ NaI,\ NaCNS$$

but not in solutions of the salts on the left.

Similarly, when the rhythmic activity of a *Limulus* ganglion has ceased in a given pure Na-salt solution, new rhythm may, or may not, be restored on transfer to another Na-salt solution, according to the effectiveness of the salt in initiating new rhythm as found above. When the rhythmic nervous discharge has stopped in NaI solution, for example, new rhythm is initiated by NaBr or any other salt on the left of NaI in the above series, but not by NaCNS. If the rhythm is stopped in NaCNS solution, new rhythm is initiated by NaI and all the other salts. When the rhythm is stopped by $NaNO_3$, however, only NaCl and Na_2SO_4 can initiate the new rhythm. This kind of experiment is best performed by abolishing the rhythm with NaCNS and NaI and then observing the recovery in the other salt solutions. For the other salts the rhythm is abolished only after a long time, and by this time the nerve is usually in bad condition. In such cases it is difficult to repeat the process of inhibition and recovery on the same nerve; for this reason the whole series has not been systematically investigated. The experiments so far performed have, however, shown uniformly that when a nerve has lost its rhythmic activity in a given salt solution, no recovery occurs in any other solution that is less effective than itself in initiating new rhythm. Here again the order of relative activity is the reverse of that found in the vertebrate tissue; some difference in the electrical properties of the structural colloids in the two cases is indicated.

In general we conclude that the effect of all pure Na-salt solutions on the activity of the ganglion is twofold: (1) a stimulating effect shown in the initiation of new rhythm and acceleration of the normal rhythm, and (2) a toxic effect shown in the decrease of amplitude of contraction and eventual standstill. The initiation and maintenance of rhythm are an important function of the Na-salts; no other salts, except Li-salts (see below), nor non-electrolytes can initiate new rhythm. Similarly, NaCl is a stimulating agent for the vertebrate heart in general (Lingle, 1900), for many invertebrate hearts and for the frog's lymph heart (Moore, 1901). It also can produce rhythmical stimulation in vertebrate muscle (Loeb, 1899) and nerve (Mathews, 1904), and it is equally important in the maintenance of the irritability of these tissues (Overton, 1902, 1904).

Other Sodium Salts: Sodium Acetate

This salt is not included in the series of neutral sodium salts since its solution has an alkaline reaction. In pure isotonic solution it has a pronounced stimulating effect on the nerve, increasing the rate several times in a few minutes; its efficiency in this respect is intermediate between that of Na_2SO_4 and that of NaCl. It differs from the other Na-salts in that it rapidly decreases the intensity of nervous discharge, the amplitude of contraction being only about one-seventh of the normal at the end of the third minute; recovery also is slow and incomplete, indicating the infliction of some permanent injury upon the nerve.

Calcium-precipitating Sodium Salts

In general, all the calcium-precipitating salts (sulphate, fluoride, tartrate, and citrate) show the same type of influence on the *Limulus*

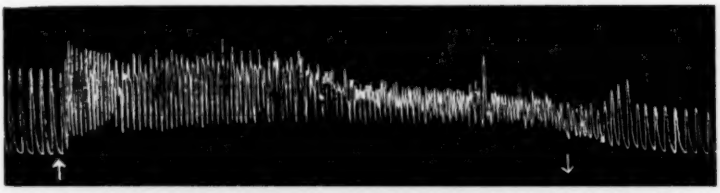


FIG. 2. Between the arrows, sea water was replaced by isotonic Na_2SO_4 for three minutes. All figures read from left to right.

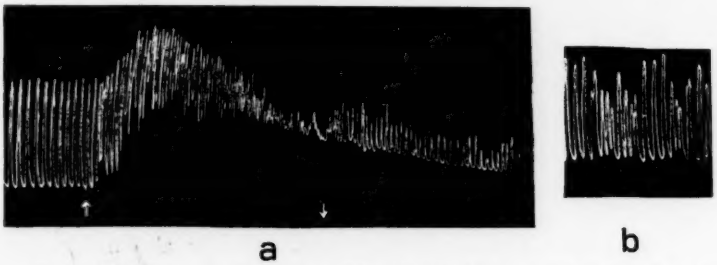


FIG. 3. (*a*) Between the arrows, Ringer's solution was replaced by isotonic sodium tartrate for two minutes.
(*b*) After 43 minutes in Ringer's solution.

nerve; all produce at first a rapid rhythmical discharge, shown by irregular contractions and tetanus in the muscle, followed by cessation and muscular relaxation within one or two minutes. The stimulating effectiveness, as shown by the rapidity of the initial rhythm and the completeness of the tetanus, is greatest with citrate and least with

sulphate; tartrate and fluoride are intermediate. The depressant or toxic effect, as measured by the time required for recovery in Ringer's solution, is greatest with tartrate; then come (in the order) fluoride, citrate, and sulphate (see Figs. 2, 3, 4, and 5).[2]

Lithium Chloride

In general LiCl has an action similar to that of NaCl, but is decidedly more toxic. The initial increase in rhythm is much greater and is followed in a few seconds by irregularity. On returning the ganglion to Ringer's solution recovery is very slow, requiring half an hour

FIG. 4. Between the arrows, Ringer's solution was replaced by isotonic sodium citrate for one minute.

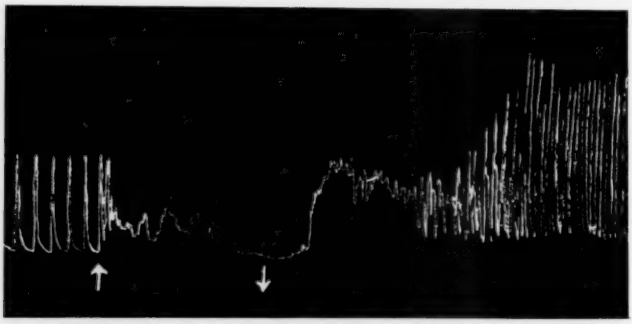


FIG. 5. Between the arrows, Ringer's solution was replaced by isotonic NaF for one minute.

or more to restore the normal rhythm. LiCl can also initiate new rhythm but is far less effective than NaCl; typically, after treating the ganglion with $CaCl_2$ for three minutes, NaCl can initiate new rhythm in one minute, while LiCl requires at least three minutes. When a nerve that has failed to recover after three minutes in LiCl is transferred into NaCl, new rhythm starts immediately. Like the other neutral sodium salts, LiCl can not replace NaCl in Ringer's solution without harmful effect.

[2] The intense stimulating action of this group of calcium-precipitating salts is also seen in frog's nerve (Mathews, 1904) and in skeletal muscles of various kinds (Garrey, 1905).

Potassium Chloride

Carlson (1906*d*) found that when the ganglion is immersed in pure isotonic KCl there is an immediate increase in the height of the first one or two contractions; this is followed by a feeble but rapid rhythm and standstill in a minute or less. A primary stimulating effect and an inhibitory effect of KCl are thus distinguishable. The stimulating effect is particularly evident when KCl is present in high concentration in Ringer's solution. Inhibition by KCl is perfectly reversible if the nerve is not subjected to its action too long. Recovery in normal Ringer's solution, however, is always slower than after Ca-inhibition, and a rapid rhythm always appears at the beginning of the recovery.

Salts of Alkali Earth Metals

Of the commonly used salts of the alkali earth metal group, chlorides of Ca, Mg, and Sr have similar action with certain differences of degree. Pure isotonic $BaCl_2$, however, as first shown on skeletal muscle by Loeb (1899), on vertebrate nerve by Mathews (1904) and on the muscle and nerve of the *Limulus* heart by Carlson (1906*d*), has an intense primary stimulating effect, somewhat like that of KCl, producing a rapid initial

TABLE IV

Average change in rate for 5 minutes (expressed as percentages of the original rate in Ringer's solution) following the addition of varying amounts of $CaCl_2$, $MgCl_2$, and $SrCl_2$ to Ca-free Ringer's solution.

	Vols. of isotonic alkali earth salt solutions added to 100 vols. isotonic NaCl and 2 vols. KCl				
	0	5	15	30	50
$MgCl_2$.....	+82	+41	+32	+ 3	−14
$CaCl_2$.....		+22	0	−11	−25
$SrCl_2$.....		+17	−12	−21	−31

rhythm followed by rapid inhibition. When the $CaCl_2$ in Ringer's solution is replaced by an equal amount of $BaCl_2$, the rhythm is increased in rate and becomes irregular.[3]

Chlorides of Ca, Mg, and Sr in pure isotonic solutions decrease both the rate and the intensity of the nervous discharge, without any indication of a primary stimulation, and arrest the rhythm in a few minutes. The order of relative effectiveness in arresting the rhythm is

$$Ca > Mg > Sr.$$

[3] $AlCl_3$ has a similar action, causing rapid initial rhythm and rapid inhibition. Solutions of $AlCl_3$ have, however, an acid reaction.

Typically, in experiments on the same nerve, Ca arrests the rhythm in about one minute, Mg in about two minutes, and Sr within three minutes. When the salts are added in varying quantities to Ringer's solution surrounding the ganglion a retardation of rhythm is also seen, but the order of relative effectiveness is different. Table IV gives the percentage change in rate of one nerve during five minutes when the ganglion is placed in Ca-free Ringer's solution (*i.e.*, a mixture of 100 vols. 0.52 M NaCl plus 2 vols. 0.52 M KCl) to which varying proportions of the

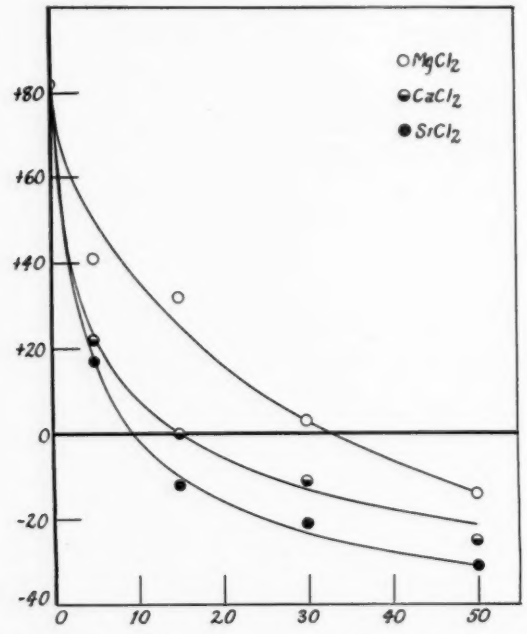


FIG. 6. Comparison of varying amounts of $MgCl_2$, $CaCl_2$, and $SrCl_2$ in Ringer's solution. Ordinate: average percentage of change in rate for five minutes. Abscissa: volumes of isotonic solutions of the alkali earth salts added to 100 vols. NaCl and 2 vols. KCl.

three salts are added, as compared with the control rate in normal Ringer's solution.

The calculations on the average percentage change in rate were made as follows. When the nerve was immersed in a solution of 100 vols. isotonic NaCl plus 2 vols. isotonic KCl, the rhythm increased from 17 beats per minute in Ringer's solution to 23, 31, 32, 33, 36 in the successive minutes. The average increase in rate for the five minutes was

from 17 to 31, or an increase of 82 per cent. All the calculations for percentage change in rate were made in the same way.

It is evident from Table IV and Fig. 6 that the relative effectiveness of the three cations in decreasing the rate under these conditions follows the order

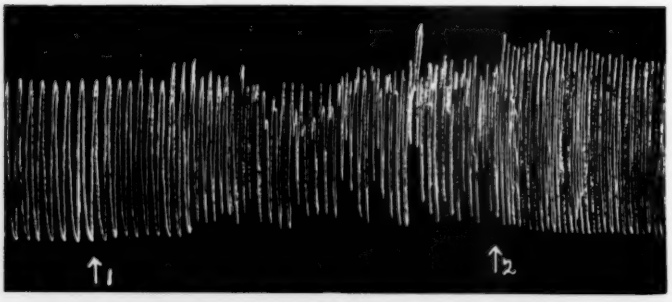
$$Sr > Ca > Mg.$$

Kisch (1927*b*) found the same series in a study of the influence of these salts on the frequency of the vertebrate heart.

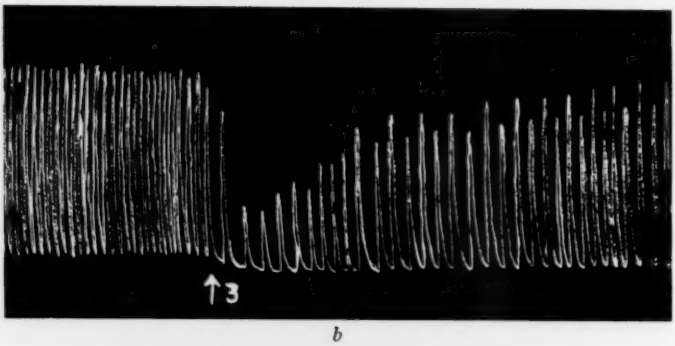
It might be expected that for each of the three salts a certain concentration could be found at which the normal rhythm would be maintained as in Ringer's solution. According to the curves of Fig. 6, about thirty volumes of $MgCl_2$, or ten volumes of $SrCl_2$, should have the same effect as the 15 volumes of $CaCl_2$. This is, however, not exactly the case. For example, in the mixture of 30 vols. $MgCl_2$, 100 vols. NaCl, and 2 vols. KCl there was actually a slight increase in rate for the first two minutes, followed by a continuous gradual decrease to a value below normal. Evidently $MgCl_2$ by itself is unable to make a balanced solution with NaCl and KCl in spite of the fact that magnesium is normally more concentrated in sea water than calcium. If $MgCl_2$ is used together with $CaCl_2$ in the normal proportion of sea water (Mg/Ca *ca.* 3), the normal rhythm is maintained quite well. This inability of $MgCl_2$ to replace $CaCl_2$ in Ringer's solution was also observed by Kisch (1929) and by Goljachowski (1932), using the vertebrate heart. In the *Limulus* ganglion $SrCl_2$ was more favorable than $MgCl_2$ as a substitute for $CaCl_2$ in Ringer's solution; yet even under the best conditions a gradual decrease in rate was also found. Apparently neither $MgCl_2$ nor $SrCl_2$ can completely replace $CaCl_2$ in Ringer's solution.

Specific Rôles of NaCl, $CaCl_2$, and KCl in Ringer's Solution and Their Antagonism

As we have seen, NaCl appears necessary for the maintenance of the ganglionic rhythm; but in pure isotonic solution of this salt the rhythm is rapid and irregular and cannot be long maintained. When to this solution $CaCl_2$ alone is added to a concentration equal to that present in normal Ringer's solution, the rhythm becomes regular and is greatly reduced in rate, although still remaining above normal, while the amplitude of the contraction is increased somewhat. But even so the normal rhythm cannot be maintained, and the amplitude gradually decreases. It is only after the addition of the normal content of KCl in Ringer's solution that the medium becomes capable of maintaining the normal rhythm for a long time (see Fig. 7). A nerve can be kept in good condition for more than six hours in this balanced solution with



a



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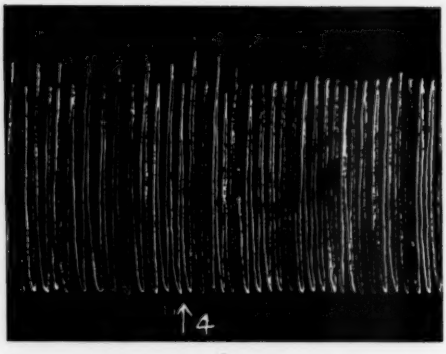

c

FIG. 7. Effect of transferring a ganglion from Ringer's solution.

(*a*) To pure isotonic NaCl at 1 for two minutes, then to a mixture of 100 vols. NaCl plus 30 vols. $CaCl_2$ at 2.

(*b*) Three minutes later, to a mixture of 100 vols. NaCl plus 30 vols. $CaCl_2$ and 4 vols. KCl at 3, showing the potassium-paradox.

(*c*) Nine minutes later, back to Ringer's solution again at 4.

only a very gradual decrease in the rate and amplitude of the heart beat. It seems, therefore, that although $CaCl_2$ antagonizes part of the toxic effect of pure NaCl the antagonism is by no means complete. Apparently the remaining part of the total effect of the NaCl is antagonized by KCl. Whether or not KCl antagonizes $CaCl_2$ in normal Ringer's solution then becomes a question. It will, however, be shown below that there is no definite antagonism between K and Ca at their normal concentration in Ringer's solution. Working with marine organisms, Rubinstein (1926, 1927) has come to a similar conclusion; namely, that NaCl has two different toxic actions, one antagonized by K and the other antagonized by Ca, and that in the ordinary balanced solution there is no antagonism between K and Ca.

A striking phenomenon is observed when a nerve is transferred from the solution containing only NaCl and $CaCl_2$ to the normal Ringer's solution containing also KCl. Instead of returning to its normal rhythm at once, either the rate or the amplitude (or both) is still further decreased, or in some cases the rhythm may cease altogether, and then after an interval return gradually to normal. This peculiar phenomenon has been described in the case of the frog's heart by Libbrecht (1920, 1921) and is called by him the potassium-paradox. It is obtained when the potassium-free perfusion fluid is exchanged for normal Ringer's solution, and is believed to be purely a potassium effect. Working on the rabbit, Busquet (1922, *a* and *b*) came to the opinion that the potassium-paradox is not a direct potassium effect but is connected with vagus stimulation. This idea has been refuted by Kisch (1927*a*). The production of potassium-paradox on the *Limulus* nerve also shows its independence of vagus action. The work on the vertebrate heart has shown that the effect depends on several factors: (*a*) on the difference in the potassium content of the two solutions (Libbrecht, 1921) (Witanowski, 1926), (*b*) on the duration of the perfusion with the K-free or K-poor solution (Kisch, 1927*a*), and (*c*) on the presence of a certain proportion of Ca (Kisch, 1927*a*). In general, the conditions are similar in the *Limulus* ganglion. The same type of potassium-paradox effect is obtained not only on changing from a K-free solution to Ringer's solution but also on changing from Ringer's solution to a solution of higher K-content. It is more readily obtained after a prolonged exposure to the K-free solution, or after repeated immersion in it. It is not obtained on changing from a pure isotonic sucrose solution to a sucrose solution containing KCl in its normal concentration in Ringer's solution, indicating that the presence of some other electrolytes is necessary. The exact conditions under which the K-paradox can be produced in the *Limulus* nerve have not been worked out in detail. It is interesting to note that Kisch (1930) has also found a similar phe-

TABLE V

Average change in rate for 5 minutes (in percentage of control rate) on addition of NaCl to 2 vols. KCl plus 15 vols. $CaCl_2$.

Vols. NaCl added	200	150	100	50	10	0
Per cent rate change	+96	+30	0	−8	−27	−36*

* Average rate for the last two minutes of rhythm (beat becomes inappreciable in five minutes).

nomenon with Ca and Sr (Ca- and Sr-paradox). The general nature of the paradox suggests that the deficiency of a certain ion (*e.g.* K-ion) produces a change in the tissue of such a kind that the subsequent addition of a small amount of the ion gives rise to its specific effect in an exaggerated form.

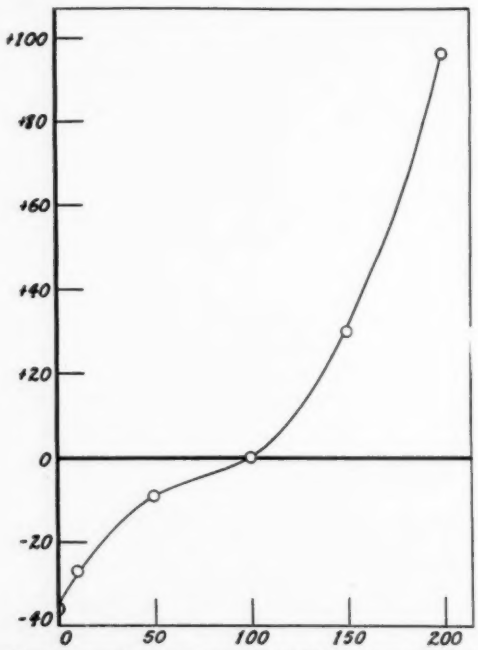

FIG. 8. Effect of varying the amount of NaCl in Ringer's solution. Ordinate: average percentage of change in rate for 15 minutes. Abscissa: vols. of isotonic NaCl added to 15 vols. $CaCl_2$ and 2 vols. KCl.

The specific rôles of the three salts in Ringer's solution can be studied by varying the concentration of each of them separately.

Effect of Varying the NaCl Content of Ringer's Solution

The normal Ringer's solution for *Limulus* heart contains 100 vols. 0.52 M NaCl, 15 vols. 0.29 M $CaCl_2$, and 2 vols. 0.52 M KCl. If, keeping constant this ratio of $CaCl_2$ and KCl, one increases the relative concentration of NaCl, a rapid rate and some irregularity appear, characteristic of the action of unbalanced NaCl. When NaCl is present in a smaller ratio than 100 vols., the rhythm is retarded, showing the effect of excess of Ca and K. Table V and Fig. 8 show the effect of varying the relative concentration of NaCl in Ringer's solution.

Effect of Varying the $CaCl_2$ Content of Ringer's Solution

In general, increase in the proportion of Ca in Ringer's solution decreases the rate of beat. Table VI gives the average results of three experiments.

The curves in Fig. 9 show the antagonism between NaCl and $CaCl_2$ beyond any doubt. As regards NaCl and $CaCl_2$, it is found that when the ratio of Ca : Na is less than 0.28 the rate is increased, indicating the predominance in the action of NaCl. When the ratio is greater than 0.28 the rate is decreased, showing a predominance of $CaCl_2$. No mixture containing only NaCl and $CaCl_2$ can maintain the normal rhythm

TABLE VI

Percentage of change in rate for 5 minutes on addition of $CaCl_2$ (1) to 100 vols. NaCl and (2) to 100 vols. NaCl plus 2 vols. KCl.

Vols. $CaCl_2$ added	(1) to 100 vols. NaCl	(2) to 100 vols. NaCl and 2 vols. KCl	(1) − (2)
0	+123	+92	31
15	+ 27	0	27
30	− 5	−25	20
50	− 30	−46	16

for long; a certain amount of KCl is indispensable. This is also shown by the lower curve in Fig. 9, giving the effect of the addition of $CaCl_2$ to a mixture of 100 vols. NaCl and 2 vols. KCl. When the proportion of $CaCl_2$ is less than 15 vols. the rate is increased; with more than 15 vols. it is decreased. The parallel course of the two curves is remarkable, showing a nearly constant influence of the KCl. There is no indication of any direct antagonism between Ca and K. Each of these cations appears to antagonize its own fraction of the toxic effect of pure NaCl, quite independently of the other.

Effect of Varying the KCl Content of Ringer's Solution

As mentioned above, in a solution containing 2 vols. 0.52 M KCl plus 100 vols. 0.52 M NaCl the rate is slower than in pure NaCl. If

more KCl is added, the rate begins to increase again (upper curve, Fig. 10). A curve of similar shape is obtained on the addition of KCl to a mixture of 100 vols. NaCl and 15 vols. $CaCl_2$ (lower curve, Fig. 10). Changing the KCl content of Ringer's solution in either direction (above or below the normal concentration of K) always results in an increase of rate. When the KCl content is decreased below normal, the rapid rate is due to the excess of NaCl; when the KCl is increased above normal, the acceleration results from the direct stimulating action of the K-ions. Accordingly we found that the toxic effect of the pure NaCl solution can be partly antagonized by the addition of 2 vols. KCl to 100 vols. NaCl;

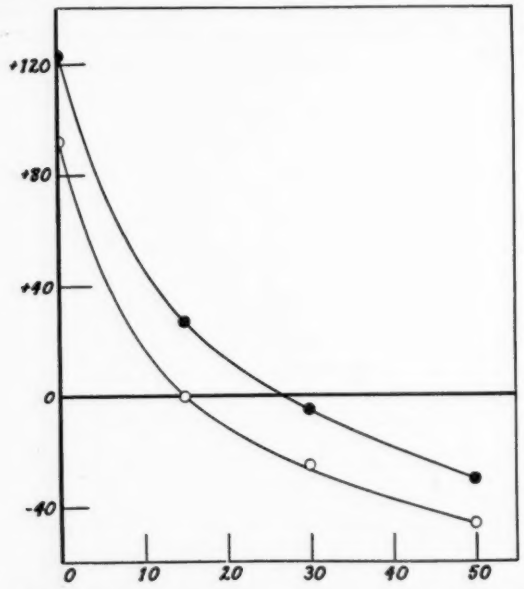

FIG. 9. Effect of $CaCl_2$. Ordinate: average percentage of change in rate for 5 minutes. Abscissa: vols. of $CaCl_2$ added (1) to 100 vols. NaCl (upper curve) and (2) to 100 vols. NaCl plus 2 vols. KCl (lower curve).

while if more KCl is added its own stimulating effect is manifested. The effects are found in the presence of the normal amount of $CaCl_2$ in Ringer's solution, indicating again the independent antagonistic actions of Ca and K in relation to NaCl (lower curve, Fig. 10). The differences of rate corresponding to the points on the two curves are seen in column four of Table VII, showing the nearly constant effect of addition of 15 vols. $CaCl_2$ to each of the solutions in column three (i.e., the

addition of a definite quantity of KCl has the same influence on the rhythm, whether it is added to the pure NaCl or to the Ca-containing NaCl solution).

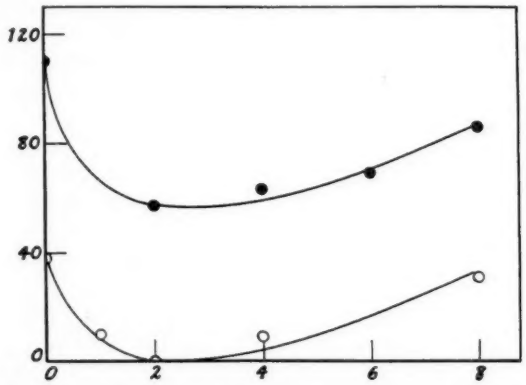

FIG. 10. Effect of KCl. Ordinate: average percentage of change in rate for 5 minutes. Abscissa: vols. KCl added (1) to 100 vols. NaCl (upper curve) and (2) to 100 vols. NaCl plus 15 vols. $CaCl_2$ (lower curve).

Effect of Varying KCl and $CaCl_2$ Together

Since an excess of KCl in Ringer's solution increases the rate and an excess of $CaCl_2$ decreases it, when both salts are present in excess we should expect to find typical antagonism. This may be tested ex-

TABLE VII

Percentage of change in rate for 5 minutes on addition of KCl (1) to 100 vols. NaCl and (2) to 100 vols. NaCl plus 15 vols. $CaCl_2$. Observations made on different nerves.

Vols. KCl added	(1) to 100 vols. NaCl	(2) to 100 vols. NaCl and 15 vols. $CaCl_2$	(1) − (2)
0	110	38	72
1	68 *	10	58
2	57	0	57
4	63	9	54
6	69	18 *	51
8	86	31	55

* Found from the curves in Fig. 10.

perimentally by adding a certain definite excess of KCl to Ringer's solution; varying additions of $CaCl_2$ are then made to this solution and the resulting changes in rate during the first five minutes are deter-

mined. When the action of K overbalances that of Ca the rate increases; when the Ca effect predominates the rate decreases. Table VIII shows the results of a series of such experiments obtained on three nerves.

TABLE VIII

Average percentage change in rate for 5 minutes on addition of $CaCl_2$ to Ringer's solution containing varying additions of KCl.

Vols. KCl added to 100 vols. Ringer	Vols. $CaCl_2$ added to 100 vols. Ringer								
	0	10	20	30	40	50	60	70	80
3	+22	− 2	−16						
6	+38	+15	+ 2	−8	−16				
9			+18	+5	− 5	−11			
12					+10	0	− 9		
18							+14	+4	−4

If the average changes in rate are plotted against the addition of $CaCl_2$, a characteristic group of curves is obtained (Fig. 11). For

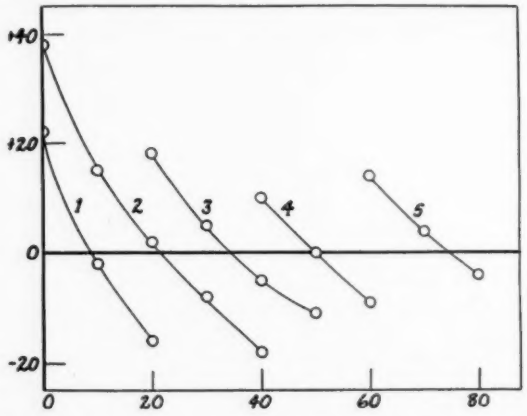

FIG. 11. Effect of addition of KCl and $CaCl_2$ together. Ordinate: average percentage of change in rate for 5 minutes. Abscissa: vols. $CaCl_2$ added to (1) 3 vols., (2) 6 vols., (3) 9 vols., (4) 12 vols. and (5) 18 vols. KCl plus 100 vols. Ringer's solution.

every concentration of KCl there should be a corresponding concentration of $CaCl_2$ at which the two salts will more or less completely antagonize each other, *i.e.* at the intersections of the curves with the abscissa. For example, in a mixture of 3 vols. KCl solution with 100

vols. Ringer's solution, about 9 vols. $CaCl_2$ should be added to antagonize the excess of KCl; with an addition of 6 vols. KCl, 21 vols. $CaCl_2$ should be required, and so on as represented in Table IX.

The Ca/K ratios in this table are obtained directly from the curves in Fig. 11. In general the ratio seems to increase as the absolute amounts of Ca and K become higher. If, however, the Ca/K ratio is calculated on the basis of their total concentration in Ringer's solution (by adding approximately 2 vols. to the added excess of KCl and 15 vols. to that of $CaCl_2$) the ratio becomes very nearly constant, the average being about 4.6. This indicates that Ca and K antagonize each other most completely at a definite ratio of 4.6, when they are present in excess in Ringer's solution. The minimal amounts of 15 vols. $CaCl_2$ and 2 vols. KCl are those required to antagonize the toxic effect of 100 vols. NaCl. Apparently only the excess of K is directly antagonized by

TABLE IX

Vols. $CaCl_2$ and KCl solutions necessary for complete antagonism of each other.

(1) added to 100 vols. Ringer			(2) added to 100 vols. NaCl		
$CaCl_2$	KCl	$CaCl_2$: KCl	$CaCl_2$	KCl	$CaCl_2$: KCl
9	3	3.0	24	5	4.8
21	6	3.5	36	8	4.5
34	9	3.8	49	11	4.5
50	12	4.2	65	14	4.6
75	18	4.2	90	20	4.5

the Ca (and vice versa). The value 4.6 should not be taken too strictly, for it does vary from nerve to nerve. Somewhere between 4 and 5 is a fair estimation on the whole. It should be added that the antagonism is not complete; a gradual decrease in the amplitude of contraction is always found. Evidently the three salts in Ringer's solution are so interrelated that it is only when they are present in the normal ratio that the solution forms a well balanced medium.

Loss of Rhythm and Loss of Irritability

Minimal Concentration of Ringer's Solution for Maintenance and Initiation of Rhythm

When a nerve is immersed in pure $CaCl_2$ solution, the rhythmic nervous discharge stops in a minute or two, but the nerve can still be stimulated by a tetanizing current, as shown by the contraction of the heart. During the next several minutes (the exact time depending on

the condition of the nerve) the irritability gradually declines and finally disappears completely, so that the nerve is no longer stimulated by a current of any strength. The dissociation of automatic rhythm and irritability is particularly obvious in some cases. Occasionally, when a nerve is dissected out, it may not show any rhythmic activity at all, although it always responds to the tetanizing current and to a pure NaCl solution. When a nerve with its irritability abolished in isotonic $CaCl_2$ is transferred into Ringer's solution, the rhythm appears gradually, together with the associated irritability. It is difficult to determine by experiment whether the irritability or the rhythmic activity returns first, for usually an automatic rhythm is started by any brief stimulation during the recovery period. In all cases, however, where definite rhythm is restored, the nerve responds to electrical stimulation; *i.e.*, automatic rhythm is always associated with irritability.

It was found by Overton (1902, 1904) that a minimal amount of NaCl is necessary for the maintenance of irritability in vertebrate muscle and nerve (about 20 vols. isotonic NaCl plus 80 vols. isotonic sucrose). The minimal amount of Ringer's solution necessary for the maintenance of irritability in the *Limulus* nerve has not yet been determined. A mixture of 33 vols. Ringer's solution plus 67 vols. isotonic sucrose solution can maintain the normal rhythm for more than one hour with only a slight increase in rate at the beginning followed by a slow progressive decline in rate and intensity. The initial stimulating effect is due to the specific action of sucrose (Carlson, 1906*c*). The unfavorable influence of the lowered salt concentration is clearly shown when after an immersion of one hour in the dilute Ringer's solution the ganglion is returned to Ringer's solution of normal concentration. The rate and intensity are at once increased. A solution containing 10 vols. per cent Ringer's solution in isotonic sucrose solution can not maintain the normal rhythm; both the rate and the intensity of the rhythmic impulses decrease gradually, and the rhythm stops in about twenty minutes (*i.e.*, in about the same time as in pure sucrose solution). Similarly, when the rhythm of a nerve is inhibited by $CaCl_2$, it recovers in a mixture of 30 vols. Ringer's solution plus 70 vols. isotonic sucrose. It appears, therefore, that this solution can maintain the normal rhythm of *Limulus* nerve for some time although not indefinitely, and can initiate new rhythm in a quiescent nerve.

Discussion

The precise conditions determining the rhythmical activity of the nerve cells in the *Limulus* ganglion are not clearly understood at present, but the analogy of Lillie's iron wire model (Lillie, 1929) suggests

that some kind of automatic rhythmic breakdown and restoration of membrane structure in these cells is the primary physical change. The rhythmic activity is closely dependent on the environmental conditions, such as temperature (Carlson, 1906*a*), the osmotic pressure of the medium (Carlson, 1906*b*), and particularly the chemical composition of the medium. The nerve appears, however, to be comparatively indifferent to decrease of oxygen tension (Newman, 1906).

One very striking property of the ganglion is the promptness with which it reacts to the presence of electrolytes; in this respect it presents a contrast with the heart muscle. While isotonic NaCl solution stimulates the ganglion almost instantaneously, the heart from which the nerve cord has been removed develops a rhythmic series of contractions only after a prolonged immersion in the same solution, *e.g.* for 30 to 45 minutes (Carlson, 1905). In vertebrate muscle and nerve a contrast of the opposite kind is seen; the frog's sartorius shows rhythmic twitchings in isotonic NaCl solution immediately after immersion (Loeb, 1899), while under the same conditions the sciatic nerve gives rhythmic impulses only after two hours or more (Mathews, 1904). The extreme sensitiveness of the *Limulus* heart ganglion to the action of salt solutions is probably to be referred chiefly to the presence of nerve cells, although the non-myelinated structure of the fibers may also be a factor.

The action of ions on the ganglion is, on the whole, quite comparable with that observed on the vertebrate heart. Some differences in the electrical properties of the structural colloids of the tissue are, however, suggested by the reversed order of the lyotropic series, described in the first part of this paper. Another difference between the two tissues is seen in the well-established antagonism between K and Ca in the vertebrate heart (Ringer, 1882, *a* and *b*), as contrasted with the independent antagonisms of KCl and of $CaCl_2$ in reference to NaCl in the *Limulus* heart. The peculiar effect of $CaCl_2$ in arresting the vertebrate heart in systole, while with KCl the arrest is in diastole, may be attributed to special structural and chemical peculiarities of this heart. In the *Limulus* heart, every contraction is the index of a train of nerve impulses originating in the ganglion. If there is no impulse from the ganglion, the heart remains in a condition of complete relaxation. $CaCl_2$ abolishes the nerve impulse without any sign of stimulation, leaving the heart muscle in a relaxed condition.

In the foregoing description, less attention has been paid to changes in amplitude of contraction than to changes in rhythm for the reason that the former show less consistency than the latter, except in those cases cited above. The changes in rate are definite and reproducible and can be conveniently used as a criterion for the evaluation and comparison of

the actions of the electrolytes. In most cases the recorded changes of rate have reference to the first five, or sometimes the first ten to fifteen, minutes after transfer from one solution to another; but these rates can not be maintained unaltered in an imperfectly balanced medium. When a nerve is immersed in an unbalanced solution for a long time, some kind of harmful effect always results and recovery is incomplete.

Summary

A Ringer's solution, containing 100 vols. 0.52 M NaCl, 15 vols. 0.29 M $CaCl_2$, and 2 vols. 0.52 M KCl, has been found satisfactory as a substitute for sea water or *Limulus* blood plasma in maintaining the normal rhythmic activity of the ganglion and heart.

Neutral sodium salts in isotonic solutions exhibit (1) a stimulating effect shown by the initiation of new rhythm in a quiescent nerve and an increase in the rate of the rhythm, and (2) a toxic effect shown by the production of irregularity and early cessation of the rhythm. The relative effectiveness of the salts in initiating new rhythm and increasing the rate follows the order

$$Na_2SO_4 > NaCl > NaNO_3 > NaBr > NaI > NaCNS.$$

The toxicity follows the reverse order.

LiCl can also initiate new rhythm but is much less effective than NaCl and more toxic.

Other sodium salts, acetate, fluoride, tartrate, and citrate, have an intense stimulating action due either to their specific toxic action, or to their calcium-precipitating property, or to the alkaline reaction of some of them.

Chlorides of Ca, Mg, and Sr inhibit the rhythm with gradual decrease in rate and intensity. The relative effectiveness in decreasing the rate follows the order

$$SrCl_2 > CaCl_2 > MgCl_2.$$

$BaCl_2$ has a primary stimulating effect but inhibits the rhythm rapidly.

Pure isotonic KCl has both a primary stimulating effect and an inhibitory effect, producing a rapid rate followed by early cessation of the rhythm. At its normal concentration in Ringer's solution, it antagonizes about one-fourth of the toxic effect of the NaCl; at higher concentrations than normal it exhibits at first a stimulating or accelerating effect and later an inhibitory effect.

$CaCl_2$ at its normal concentration in Ringer's solution antagonizes about three-fourths of the toxic effect of the NaCl; at higher concen-

trations than normal it produces an inhibitory effect, decreasing the rate and amplitude.

Ca and K antagonize each other at a definite proportion (about 4 or 5 to 1) when both are present in excess in Ringer's solution.

Potassium-paradox has been observed on changing from a potassium-free solution to normal Ringer's solution.

The normal rhythm can be maintained by Ringer's solution of 30 per cent normal concentration (made isotonic by sucrose) and new rhythm can be initiated by the same solution. The rhythmic activity of the ganglion is more readily abolished than its responsiveness to electrical stimulation.

The author wishes to express his gratitude to Dr. R. S. Lillie for much help and valuable advice, to Dr. W. E. Garrey for demonstrating his method of opening *Limulus*, to Dr. C. Ladd Prosser and Mr. A. L. Chute for apparatus and chemicals used in this work, to Mr. J. C. Bridges for calling attention to the phenomenon of K-paradox in the vertebrate heart, and to Mr. T. C. Butler for much help in some of the preliminary experiments.

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THE EQUILIBRIUM BETWEEN HEMOGLOBIN AND OXYGEN IN THE BLOOD OF CERTAIN FISHES

ARDA ALDEN GREEN AND RAYMOND W. ROOT
THE WOODS HOLE OCEANOGRAPHIC INSTITUTION [1]

In studies made on the hemoglobin of certain teleost fishes Root (1931) found that carbon dioxide had a remarkable effect on the ability of the blood to combine with oxygen. As in the case of mammalian blood, the addition of acid decreases the amount of oxygen combined with the hemoglobin at any given oxygen tension, but in these fish bloods the addition of acid decreases also the total amount of oxygen that can be combined even when the oxygen tension is increased to that of air. As a consequence the suggestion was made that the prosthetic groups in the hemoglobin molecule were differentially affected by the presence of acid, some being inactivated entirely so that they no longer combined with oxygen. Since confirmation of this suggestion promised to throw new light on the theory of the equilibrium between oxygen and hemoglobin, the phenomenon has been examined in greater detail.

In the present study we have examined the blood of the tautog, *Tautoga onitis* Linnæus, the goosefish, *Lophius piscatorius* Linnæus, and the toadfish, *Opsanus tau* (Linnæus). We have confirmed the observation of Root and shown that the amount of oxygen combined is decreased in the presence of acid even though the oxygen tension is raised to one atmosphere. In addition, the oxygen dissociation curves have been studied throughout the range of hydrogen ion concentration in which the phenomenon is described. These curves are remarkable as to shape, especially those of the toadfish, which have a distinct undulatory character. Another characteristic is a change in the shape of the curves with a change in pH. In alkaline solutions the curves for the goosefish and for the tautog are sigmoid whereas in more acid solutions the curves are rectangular hyperbolæ. Thus in these teleost fishes we find certain unique characteristics exhibited by the oxygen dissociation curves. Such peculiarities must be accounted for by any acceptable general theory of the union of oxygen with hemoglobins.

METHODS

The blood of the goosefish was obtained from the bulbus or sinus venosus, and was removed from the animal shortly after it was taken

[1] Contribution No. 7.

from the commercial fish traps. On account of the low hemoglobin concentration of the blood of this fish it was customary to centrifuge the blood and draw off plasma equal to about one-third the total volume, before making analyses. The other fishes were maintained at the laboratory in tanks, and were bled from the gills by means of a hypodermic syringe. Lithium oxalate was used as an anticoagulant. Samples of blood were necessarily combined in any given species of fish and the reserve supply kept on ice. In most cases the blood was used within 12 hours. In the case of the goosefish it was possible to keep a sample of blood several days without the appearance of methemoglobin.

The usual methods of obtaining oxygen dissociation curves were employed as described by Dill (1928). Carbon dioxide and oxygen analyses on the blood were done simultaneously as described by Root (1931). The analysis of gas mixtures containing over 30 per cent carbon dioxide or oxygen was made possible by a special modification of the Haldane apparatus. Gas pressures in the tonometers were calculated by use of the formula of Bock, Field, and Adair (1924). Samples of blood were equilibrated at a temperature of 25° C. for 15 minutes in a water bath. Nitrogen was used as the inactive gas in the tonometers. It is essential to point out that but one tonometer was equilibrated at a time and analyses followed directly after equilibration. This procedure is quite necessary in fish blood, since it possesses active, nucleated erythrocytes (Dill, Edwards, and Florkin, 1932). Duplicate analyses, both of the gases in the blood and in the tonometer, were made for all established points.

Since the oxygen combined by these bloods is diminished in the presence of acid, the oxygen capacities were always determined on blood to which a small amount of powdered sodium bicarbonate had been added. This blood was then equilibrated either in air or in oxygen and the figure obtained after dissolved oxygen was subtracted was taken to be the real oxygen capacity of the hemoglobin.

A few of the more acid samples turned brownish in color and the formation of methemoglobin was suspected. In such cases the oxygen capacity of a duplicate sample of the blood was determined directly after each equilibration by using sodium bicarbonate to bring the blood once more to an alkaline reaction and re-equilibrating with air. Whenever the hemoglobin had been irreversibly inactivated the percentage saturation was calculated by using the oxygen capacity as determined after equilibration rather than the original value.

All the data expressing the quantity of oxygen in blood were corrected for dissolved oxygen. In the case of toadfish and goosefish

the solubility coefficient for oxygen was experimentally established as 2.70 vols. per cent for 760 mm. O_2 at 25° C. This figure was assumed to hold in the case of the tautog.

In studies of the effect of acid on the ability of blood to combine with oxygen, samples of blood were equilibrated in oxygen or air to which had been added various amounts of carbon dioxide. When acidities were desired greater than could be obtained in this way a few drops of 4 per cent lactic acid were added.

The pH of fish blood is not easy to control and great difficulties were experienced in trying to establish dissociation curves at constant pH. On account of the great change in acidity of the hemoglobin with degree of oxygenation the use of a constant carbon dioxide tension does not lead to a constant pH at all oxygen tensions. This difficulty was overcome by grading the amount of carbon dioxide added and, in some cases, by adding a small amount of sodium bicarbonate. In this way it was possible to obtain points calculated to have approximately the same pH values at all degrees of oxygenation. The pH of the blood was calculated from gasometric data, using the Henderson-Hasselbalch equation and assuming pK 6.22 for whole blood at 25° C. Dissolved carbon dioxide was calculated by using Bohr's solubility coefficient slightly modified in a manner prescribed by Peters, Bulger, and Eisenman (1924) to allow for differences in corpuscular volume. The final factors used to calculate the volume percentage of dissolved CO_2 were 0.102 pCO_2 for toadfish, and 0.1 pCO_2 for the other fish bloods. It must be remembered that the electrolyte equilibrium between cells and plasma is completely ignored and the pH is calculated from determinations of the carbon dioxide equilibrium of whole blood rather than that of either plasma or cells.

The Apparent Loss of Oxygen Capacity in Acid Solutions

Root (1931) found that the amount of oxygen combined with certain fish bloods at a pressure of oxygen equivalent to that of air depended upon the acidity of the blood. The addition of very small amounts of carbon dioxide (even 5 or 10 mm.) or of lactic acid, appreciably decreased the oxygen bound. This effect is reversible, for if the blood equilibrated under acid conditions is again made alkaline it regains its original combining capacity.

It seemed to us that this phenomenon might be merely a pronounced "Bohr" effect (Bohr, Hasselbalch, and Krogh, 1904) in which the acid altered the oxygen dissociation constant to such an extent that the oxygen tension of air was insufficient to completely saturate the hemoglobin. If higher oxygen tensions had been used, saturation

might have been complete and the oxygen capacity undiminished. To test this possibility fish blood was equilibrated with oxygen containing the requisite quantity of carbon dioxide. Since the total pressure was about one atmosphere, the oxygen tension of these gas mixtures decreased with increasing carbon dioxide concentration. For this reason lactic acid was added on some of the more acid samples to enable a relatively high acidity to be reached without too greatly diluting the oxygen with carbon dioxide.

The results upon the blood of the goosefish, the tautog, and the toadfish in both air and oxygen at 25° C. are represented in Fig. 1.

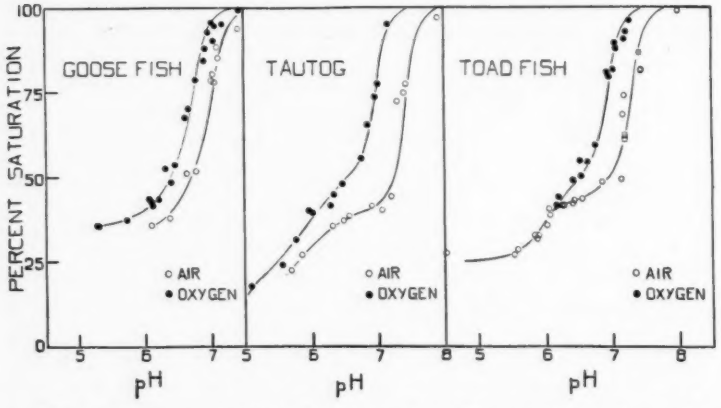

FIG. 1. The oxygen combined in fish bloods equilibrated with varying tensions of carbon dioxide and oxygen or of carbon dioxide and an oxygen tension approximating that of air.

The oxygen tension of the more acid points in the curves of the toadfish is about 500 mm., of the tautog 650 mm., and of the goosefish 350 mm. Except in the acid ranges of the toadfish blood, the samples equilibrated with oxygen combined with more oxygen than those treated with air. But it still remains a question as to whether the oxygen dissociation curves have shifted or whether the amount of oxygen which can be combined has been decreased. It seemed desirable, consequently, to determine the entire oxygen dissociation curves at various hydrogen ion concentrations in the hope that their analysis would enable us to determine the relative part played by these two phenomena.

EQUATIONS FOR OXYGEN-HEMOGLOBIN EQUILIBRIUM AT CONSTANT pH

There have been three types of equations describing oxygen dissociation which have proved useful for our purpose. The first of these

is that of Hill (1910). The fraction of hemoglobin combined with oxygen, in the following manner:

$$Y = \frac{Kx^n}{1 + Kx^n}, \tag{1}$$

where x is the oxygen concentration expressed as pressure in mm. Hg. Equation (1) may be derived from the mass law on the assumption that n is the number of molecules of oxygen uniting with each molecule of hemoglobin.

According to Adair's hypothesis, each molecule of hemoglobin combines with four molecules of oxygen and the molecule $Hb(O_2)_4$ is built up and broken down in stages, thus:

$$Y = \frac{0.25K_1(O_2) + 0.5K_2(O_2)^2 + 0.75K_3(O_2)^3 + K_4(O_2)^4}{1 + K_1(O_2) + K_2(O_2)^2 + K_3(O_2)^3 + K_4(O_2)^4}, \tag{2}$$

in which K_1, K_2, K_3, and K_4 are products of mass law equilibrium constants. This equation has been successfully used to describe the curves for man (Adair, 1925), the horse (Ferry and Green, 1929), and the sheep (Ferry and Pappenheimer, 1929).

Another expression has been suggested by Redfield (Ferry and Green, 1929, p. 194) and has been found useful in describing the oxygen dissociation curves of a number of bloods containing hemocyanin which have a distinct undulatory character (Redfield, 1933). It may be assumed that the respiratory protein consists of two or more components, each of which reacts with oxygen independently of the others and reaches an equilibrium described by a distinct oxygen dissociation curve. Further, each component reacts with oxygen in accordance with Hill's equation (equation 1) but the components are each characterized by a different value of n. If the oxygen dissociation constants of the forms characterized by values of n of 1.0, 2.0, 3.0, 4.0, etc. are designated by K_1, K_2, K_3, K_4, etc., and the fraction of the total oxygen bound by each of these forms as α_1, α_2, α_3, α_4, etc., the fraction of the total respiratory protein present in the oxygenated condition, Y, is given by the equations:

$$Y = \frac{\alpha_1 K_1 x^1}{1 + K_1 x^1} + \frac{\alpha_2 K_2 x^2}{1 + K_2 x^2} + \frac{\alpha_3 K_3 x^3}{1 + K_3 x^3} + \frac{\alpha_4 K_4 x^4}{1 + K_4 x^4} \cdots.$$

In applying this expression to the fish bloods which we have studied, it is not necessary to employ more than the first two terms so that the expression becomes

$$Y = \alpha_1 \frac{K_1(O_2)}{1 + K_1(O_2)} + \alpha_2 \frac{K_2(O_2)^2}{1 + K_2(O_2)^2}. \tag{3}$$

TABLE I

Oxygen Dissociation of Toadfish Blood Equilibrated at 25° C.

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
17	8.76	1.35	1.32	6.98	18.9	38.8	67.3	7.42
	19.4	2.57	2.50		35.8	36.3	64.7	7.44
	25.3	2.96	2.87		41.1	34.7	65.8	7.47
	44.25	3.71	3.55		50.9	36.7	67.0	7.45
	69.8	4.47	4.22		60.5	34.3	62.9	7.45
	80.0	4.62	4.34		62.2	36.1	65.1	7.44
	90.9	5.11	4.79		68.6	34.0	63.0	7.45
	107.0	5.26	4.88		70.0	35.1	64.2	7.45
	115.0	5.28	4.87		69.8	36.4	63.2	7.43
	117.5	5.30	4.88		69.9	34.9	64.0	7.45
	134.2	5.81	5.33		76.4	34.75	63.2	7.45
	151.0	6.21	5.68		81.4	33.85	61.55	7.44
	172.0	6.56	5.95		85.3	36.3	61.4	7.41
	191.0	6.72	6.04		86.6	34.1	63.5	7.44
	261.5	7.27	6.34		90.8	35.4	58.2	7.40
19 †	2.69	0.47	0.46	7.21	6.39	46.3	48.2	7.18
	14.7	1.95	1.90		26.3	45.2	47.5	7.19
	26.9	2.81	2.72		37.7	42.5	47.4	7.22
	53.8	3.41	3.22		44.7	41.4	46.35	7.22
	80.0	3.77	3.49		48.4	41.3	46.85	7.22
	102.0	4.16	3.80		52.7	42.0	46.0	7.21
	131.4	4.54	4.07		56.5	41.0	46.2	7.21
	147.0	4.94	4.42		61.3	43.0	45.1	7.19
	163.4	5.24	4.66		65.2	38.9	44.7	7.23
	209.5	5.79	5.05		70.0	43.8	44.1	7.17
	247.0	6.31	5.44		75.5	40.75	42.8	7.19
	322.0	6.73	5.59		78.2	40.25	43.0	7.20
	410.0	7.44	5.99		83.8	39.5	41.5	7.19
	512.0	8.01	6.19		86.5	39.8	41.8	7.19
	723.0	9.10	6.53		90.6	39.2	41.2	7.19
18	4.23	0.71	0.69	7.15	9.70	35.6	23.6	6.96
	16.7	1.97	1.91		26.7	35.2	23.3	6.96
	25.1	2.53	2.44		34.1	34.9	22.5	6.95
	53.2	3.50	3.31		45.3	34.2	22.6	6.96
	107.1	3.89	3.51		49.2	34.4	23.05	6.96
	137.7	3.98	3.49		48.8	45.7	24.7	6.83
	150.0	4.11	3.58		50.0	32.0	22.2	6.98
	225.0	4.66	3.86		53.0	31.6	22.2	6.99
	291.0	5.18	4.15		58.0	34.2	24.8	7.01
	352.0	5.59	4.34		61.0	34.2	22.3	6.95
	388.0	6.06	4.68		65.5	33.6	23.6	6.99
	461.0	6.54	4.90		68.6	34.4	23.5	6.98
	500.0	6.93	5.16		72.1	33.3	21.4	6.94
	729.0	8.37	5.68		79.5	34.6	22.4	6.96

TABLE I—*Continued*

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
6	4.23	0.65	0.64	6.04	10.6	51.85	28.3	6.86
	6.96	0.86	0.84		13.9	47.75	27.2	6.88
	16.65	1.70	1.65		27.2	50.7	27.2	6.85
	33.9	2.30	2.19		36.2	59.6	29.0	6.80
	69.9	2.76	2.53		41.8	53.9	26.9	6.81
	96.6	3.05	2.73		45.2	53.9	32.5	6.92
9	200.	3.62	2.91	6.20	47.0	46.3	26.2	6.89
	285.	3.98	2.97		48.0	47.7	30.3	6.94
	331.	4.30	3.13		50.5	46.6	25.9	6.88
	345.	4.60	3.38		54.5	47.3	25.2	6.86
	402.	5.02	3.60		58.0	44.5	22.7	6.83
	481.	5.32	3.61		58.2	46.6	25.8	6.87
	580.	5.59	3.54		57.1	45.5	26.9	6.91
	649.	5.94	3.64		58.7	46.1	24.5	6.86
5	4.90	0.61	0.61	6.16	9.9	120.2	39.55	6.57
	8.17	0.89	0.88		14.3	119.4	39.65	6.57
	12.74	1.18	1.16		18.8	110.8	38.25	6.60
	20.82	1.65	1.57		25.5	129.5	40.55	6.54
	36.14	2.05	1.90		30.8	123.0	40.00	6.56
	69.2	2.56	2.33		37.8	125.5	41.05	6.56
14	6.05	0.665	0.64	6.06	10.7	100.7	18.68	6.13
	13.10	1.015	0.97		16.0	100.2	18.40	6.12
	23.45	1.38	1.30		21.5	100.5	18.33	6.12
	33.7	1.63	1.51		24.9	97.2	18.10	6.13
	40.8	1.90	1.76		29.0	98.1	17.80	6.11
	48.7	2.02	1.85		30.5	97.7	17.48	6.10
	58.6	2.21	2.00		33.0	98.8	17.95	6.11
	91.0	2.44	2.12		35.1	97.4	18.00	6.13
	140.6	2.84	2.34		38.6	105.3	18.17	6.06

* Each experiment number represents measurements made on a single collection of blood.

† Sodium bicarbonate added to the blood.

In order to take account of the apparent inability of all of the hemoglobin to combine with oxygen, which characterizes the more acid samples, it is necessary to introduce a term α_0, equal to the fraction of total hemoglobin incapable of combining with oxygen. It follows that

$$\alpha_0 + \alpha_1 + \alpha_2 = 1.$$

THE OXYGEN DISSOCIATION CURVES OF TOADFISH BLOOD

The results of the experiments on the blood of the toadfish are given in Table I and the oxygen dissociation curves at constant pH are shown in Fig. 2.

These curves may be observed to have certain interesting characteristics regardless of the theory or of the equations used to describe the oxygen-hemoglobin equilibrium. The first of these is a difference in the shape of the curves at different hydrogen ion concentrations. In mammalian blood, on the contrary, curves at constant carbon dioxide tension or at constant pH are apparently of one family having the same shape.

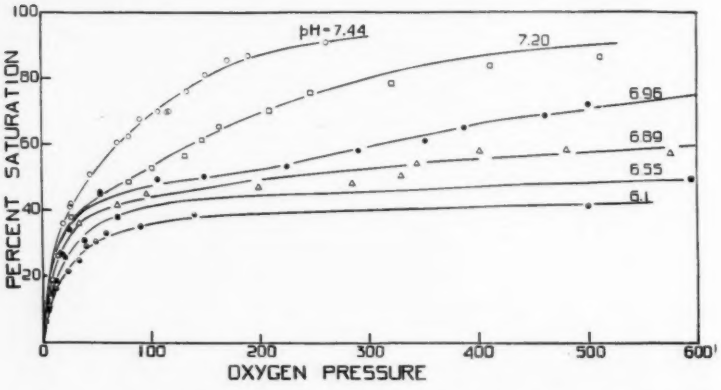

FIG. 2. Oxygen dissociation curves at constant pH of the blood of the toadfish. The curves have been drawn according to equation (3) using the following constants:

pH	α_1	α_2	α_3	K_1	$K_2 \times 10^4$	$1/K_1$	$1/K_2$
7.44	.5	.5	0	.11	.826	9	110^2
7.20	.5	.5	0	.10	.186	10	232^2
6.96	.5	.48	.02	.10	.033	10	548^2
6.89	.5	.2	.3	.071	.033	14	548^2
6.55	.5	0	.5	.0475		21	
6.1	.43	0	.57	.0475		21	

The data may be equally well described by equation (2) when the following values for the constants are used:

pH	K_1	$K_2 \times 10^2$	$K_3 \times 10^4$	$K_4 \times 10^6$
7.44	.2	1.0	.01	1.0
7.20	.1	.6	.04	.10
6.96	.1	.5	.001	.015
6.89	.1	.4	.001	.004
6.55	.1	.2	.001	.0001

A second effect of pH on these curves is a shift to the right with increasing acidity, a phenomenon well established for various mammalian bloods. On the other hand, the change in the position of the curve with change in pH is almost insignificant in the blood of the elasmobranch, *Raia ocellata* (Dill, Edwards, and Florkin, 1932). In these marine teleosts, and especially in the toadfish, the effect is very marked. As a result the curves determined at pH 7.2, 6.96, and 6.89 are not completely saturated even in the presence of 500 mm. O_2 and the shape of the curves indicates that more oxygen would be combined at higher oxygen tensions.

This is an explanation of a part of the decrease in oxygen capacity described by Root (1931). It should be noted, however, that the curves determined at pH 6.55 and 6.1 are practically flat at tensions of oxygen above 150 mm. which suggests that saturation is complete but that a part of the hemoglobin is incapable of combining with oxygen.

The next important feature of the curves is their peculiar shape. Some of them are not simple hyperbolic or sigmoid curves but are undulatory in character, having two regions concave to the abscissa. Similar types of curves have been found for the blood of some birds (Wastl and Leiner, 1931) and of certain animals containing hemocyanin (Redfield, 1933). The break between the two regions of the curves occurs at about 50 per cent saturation, and the more acid curves, in which the combined oxygen has become constant, do not rise above 50 per cent saturation. This characteristic of the curves suggests that the oxygenation of the hemoglobin consists of two steps, or that there are two components to the curves representing the behavior of different fractions of the pigment present in equal quantities. Theoretical treatment of these possibilities is provided by the equations given above.

The equation (1) developed by Hill applies only to simple curves, hyperbolic or sigmoid in shape, and thus could be used to describe only the most acid curves and these only if the decrease in oxygen capacity is assumed to be real so that higher oxygen tensions would not increase the oxygen bound.

Although Adair's theory in which the hemoglobin combines with oxygen in steps was derived for sigmoid curves, equation (2) may be used to describe the oxygen dissociation curves of the toadfish. By making K_3 and K_4 sufficiently small, the terms in which they appear become ineffective at the oxygen tensions used and the curve becomes flat at one half saturation, as in the experiment at pH 6.55. According to this treatment the decrease in oxygen capacity is only apparent and

the effect really one on the magnitude of the constants and thus a "Bohr" effect. However, the curve at pH 6.1 is indubitably flat over a long range at less than 50 per cent saturation, that is, at 43 per cent, and it is also apparent from Fig. 1 that a further increase in acidity would further decrease the oxygen bound. Such curves can only be derived from the Adair equation by assuming a true loss in oxygen capacity, thus necessitating the introduction of a new constant to describe this loss.

The curves may be described equally well in terms of Redfield's equation which employs fewer constants. The smooth curves in Fig. 2 are drawn according to equation (3). When the curves have not flattened out at one atmosphere of oxygen, the fraction of hemoglobin incapable of combining with oxygen must be calculated from the shape of the dissociation curves at lower tensions and this unfortunately gives an additional degree of freedom in analyzing some of the curves.

The difference between the values for K_1 and K_2 gives the undulatory character to the curves. In some cases the hemoglobin combining with oxygen according to the first constant is almost completely saturated before the second inflection begins. By taking α_1 and α_2 each = 0.50, which implies that the amount of hemoglobin acting as though it combined with one molecule of oxygen at a time exactly equals that combining with two molecules, the flat places in the curves or the points where there is a change of inflection occur at about one half saturation.

The decrease in oxygen capacity takes place first at the expense of the component behaving as though $n = 2$. The value of α_1, the amount of hemoglobin behaving according to the first term of the equation, remains constant at 0.50 throughout the pH range until the amount of hemoglobin capable of combining with oxygen has been reduced to 50 per cent of the whole. Thus, the curve at pH 6.55, in which the capacity is 50 per cent of the total oxygen-combining power, is a rectangular hyperbola. Further addition of acid reduces the capacity but the curve is still of the same shape and has the same value for the constant K_1. This implies that the effect described by Root comprises a real inactivation of the hemoglobin and is due only in part to a greatly exaggerated "Bohr" effect.

All three treatments have certain common implications. The description of the curves in accordance with either equation (1), (2), or (3) requires the assumption that a portion of the hemoglobin has lost its ability to combine with oxygen in the case of the most acid solution. In the use of equation (3) it is implied that the prosthetic

groups responsible for the two components of the curves are divided in equal amounts. This condition could be met if there were two forms of hemoglobin present, but the probability of two independent substances existing in such exact proportion is rather small. It is much simpler to postulate the existence of a number of oxygen-combining groups on one molecule, one half of which behave in one way and one half in the other. Since one half of the group behave as though they combined with oxygen in pairs, at least four prosthetic groups must be attributed to the molecule. In this regard the implications regarding the structure of the hemoglobin molecule are the same as those postulated by Adair in deriving equation (2).

The Oxygen Dissociation Curves of the Blood of the Tautog and of the Goosefish

The blood of the tautog and of the goosefish may be considered together since they exhibit similar characteristics. The data are presented in Tables II and III.

The most important characteristic of these curves is a change in the shape of the curve with a change in pH. Equation (1) may be transformed to the logarithmic form:

$$\text{Log}\frac{HbO_2}{Hb} = \log K + n \log pO_2.$$

When log HbO_2/Hb is plotted against log pO_2, the resulting curve is a straight line, the slope of which is equal to n. The data for these two fish bloods have been calculated in this manner and the results plotted in Fig. 3. In both cases the most alkaline curves are straight lines and the slope is 2.0, that is, Hill's equation is applicable and n is 2.0. In both cases, also, the most acid curves are straight lines but here n is 1.0 and consequently the oxygen dissociation curves are rectangular hyperbolæ.

In both these bloods the oxygen-combining power is decreased in the more acid solutions. In the tautog the curve has changed to a rectangular hyperbola ($n = 1.0$) at pH 7.2. At this reaction the oxygen capacity is not appreciably lowered, so that the change in shape is independent of this phenomenon. In the goosefish the decrease in oxygen capacity begins to appear before the curves have assumed a shape characterized by $n = 1$, and in applying Hill's equation it is necessary to make allowances for the decreased oxygen capacity of the blood, as was done in the case of toadfish blood (p. 391). At pH 6.84, 6.80, and 6.1 the oxygen capacities are assumed to be 88, 80, and 54 per cent respectively, of those obtaining in alkaline solutions.

TABLE II

Oxygen Dissociation of Goosefish Blood Equilibrated at 25° C.

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
3	2.77	2.80	2.79	8.40	33.2	2.53	24.1	8.20
	7.08	5.70	5.68		67.5	3.16	25.2	8.11
	10.4	7.02	6.98		83.0	2.14	22.9	8.23
	15.94	7.90	7.84		93.3	2.41	23.2	8.20
	66.9	8.74	8.50		101.0	2.76	24.2	8.15
4 †	5.26	5.07	5.05	8.59	58.8	6.36	51.3	8.12
	6.79	5.64	5.62		65.5	8.54	56.2	8.03
	7.74	6.42	6.39		74.4	6.17	50.4	8.13
	7.89	6.35	6.32		73.6	7.23	54.3	8.09
	12.33	7.50	7.46		86.8	7.20	53.9	8.09
	14.58	7.96	7.91		92.2	6.29	52.0	8.15
	25.1	8.32	8.23		95.8	6.22	51.2	8.13
3	3.19	0.93	0.93	8.40	11.1	0.98	2.79	7.65
	21.3	5.12	5.04		60.0	0.91	2.8	7.52
	25.8	5.82	5.73		68.2	0.93	2.07	7.55
	32.3	6.36	6.25		74.3	0.61	1.6	7.62
	58.0	7.54	7.33		87.4	1.10	2.3	7.52
4 †	9.86	1.22	1.18	8.59	13.7	48.3	39.5	7.08
	25.7	3.09	3.00		35.0	45.6	37.5	7.08
	31.35	3.67	3.56		41.4	47.2	37.3	7.06
	39.85	4.45	4.31		50.3	43.5	35.0	7.07
	68.7	5.92	5.68		66.2	42.3	33.0	7.07
	109.6	7.36	6.97		81.0	36.4	30.1	7.08
	151.	8.11	7.58		88.3	33.2	28.4	7.10
	182.	8.65	8.00		93.3	33.8	28.3	7.09
4 †	12.0	1.41	1.37	8.59	15.9	49.6	39.6	7.06
	25.1	2.76	2.67		31.1	49.7	39.2	7.06
	37.4	3.45	3.32		38.6	50.7	37.8	7.03
	54.4	4.37	4.18		48.7	49.6	37.7	7.04
	104.6	7.00	6.63		77.3	42.4	32.8	7.05
	149.5	7.22	6.69		77.9	41.3	32.6	7.06
	148.2	7.43	6.90		80.3	41.1	31.5	7.04
	228.	8.38	7.57		88.3	41.2	30.3	7.03
3	4.05	0.55	0.54	8.40	6.45	50.7	26.0	6.84
	19.8	1.52	1.45		17.3	50.0	25.7	6.84
	27.35	2.15	2.05		24.4	50.7	26.2	6.84
	59.9	3.57	3.36		40.0	50.8	24.9	6.81
	102.6	4.31	3.95		47.2	50.3	24.9	6.82
	197.5	5.99	5.29		62.8	49.6	23.3	6.80
	303.5	6.92	5.84	8.28	70.5	46.7	22.0	6.79
	593.	8.73	6.62	8.19	80.7	46.4	20.7	6.76
	732.	9.05	6.45	7.94	81.3	48.5	21.3	6.75

TABLE II—*Continued*

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
4	7.0	0.72	0.70	8.59	8.15	41.0	19.8	6.80
	12.8	1.12	1.07		12.5	41.8	20.2	6.80
	34.5	2.22	2.10		24.5	39.4	19.5	6.82
	80.4	3.87	3.58		41.7	38.8	16.6	6.74
	106.5	4.39	4.02		46.8	37.6	18.1	6.80
	150.	5.01	4.48		52.1	34.9	16.6	6.78
	250.	6.34	5.45		63.5	31.2	14.1	6.77
	743.	9.08	6.44		75.0	25.1	11.6	6.78
3	34.6	1.88	1.76	8.40	20.9	390.	70.6	6.13
	64.3	2.24	2.00	8.32	23.8	374.	67.2	6.12
	106.	2.88	2.50	8.03	31.2	404.	72.0	6.11
	150.	3.39	2.86	7.96	35.8	374.	66.6	6.11
	378.	4.35	3.01	7.24	41.6	377.	68.7	6.13

* Each experiment number represents measurements made on a single collection of blood.

† Sodium bicarbonate added to the blood.

The curves at intermediate pH values are such that n lies between 1 and 2. The curve for the tautog at pH 7.4 is apparently a straight line of slope 1.3 so that equation (1) is applicable whereas the intermediate goosefish curves would seem to be slightly curved, indicating that a more complicated equation would give a more satisfactory description.

There is no question but that equation (2) could be used to describe these curves but their simple character renders the application of such a complicated expression of little interest.

Equation (3) may also be successfully applied here. The smooth curves in Figs. 4 and 5 are drawn according to this equation using the constants given in the legends.

Thus the dissociation curves of the tautog and the goosefish are so simple in character that they may be described by equations based on any one of the theories of the mode of combination between oxygen and hemoglobin, and in themselves give no basis for a preference of one theory over another.

These curves leave little doubt that the decrease in oxygen in acid solution is really due to a loss in ability to combine with oxygen, and not to a "Bohr effect," for certain of the curves have the same dissociation constant and differ only by the total amount of oxygen with which they are able to combine when the acidity is changed. The

TABLE III

Oxygen Dissociation of Tautog Blood Equilibrated at 25° C.

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
6 †	9.56	1.45	1.42	9.66	14.7	12.60	56.1	7.86
	13.13	3.04	2.99		30.9	12.5	55.0	7.85
	14.23	3.07	3.02		31.3	11.80	55.1	7.88
	21.8	4.90	4.82		50.0	10.97	52.4	7.89
	38.7	7.07	6.93		71.7	10.02	50.2	7.91
	42.9	7.73	7.58		78.5	10.35	50.1	7.90
	84.2	9.09	8.79		90.7	9.59	49.1	7.92
2	12.9	1.17	1.12	8.74	12.7	9.13	15.05	7.41
	15.2	1.52	1.47		16.7	7.62	13.50	7.45
	22.6	1.97	1.89		21.5	7.89	14.35	7.46
	46.7	4.24	4.06		46.1	8.08	12.33	7.38
	70.1	5.10	4.85		55.0	7.98	12.12	7.38
	155.	7.33	6.80		77.2	5.73	9.50	7.41
	276.	8.92	7.95		90.2	5.11	8.06	7.40
4	16.4	1.38	1.32	9.39	14.2	25.8	26.1	7.18
	52.5	2.89	2.71		29.1	26.3	26.3	7.17
	109.5	4.89	4.50		47.9	19.86	22.3	7.23
	152.5	5.62	5.08		54.1	21.1	22.2	7.20
	214.5	6.45	5.69		61.1	18.8	20.8	7.22
	268.	7.08	6.13		65.9	19.3	20.5	7.20
	381.	8.37	7.02		75.5	16.8	17.8	7.20
3	13.9	1.10	1.05	7.66	13.7	118.8	36.8	6.54
	30.5	1.78	1.67		21.8	116.6	36.2	6.54
	53.8	2.30	2.11		27.5	118.5	36.5	6.54
	87.9	2.59	2.28		29.7	137.4	38.9	6.48
	124.7	3.14	2.70		35.3	130.2	36.6	6.48
	147.0	3.46	2.94		38.4	106.8	33.6	6.55
	150.8	3.36	2.83		37.0	130.7	36.8	6.48
	163.8	3.61	3.03		39.5	117.6	36.5	6.54
	309.0	4.62	3.52		45.8	123.0	36.4	6.51
	625.0	5.91	3.69		48.0	134.2	37.6	6.46
5 ‡	23.3	0.82	0.74	9.54	7.74	107.3	14.33	5.73
	39.6	1.34	1.20	8.38	14.3	104.5	15.10	5.87
	54.2	1.39	1.20	7.87	15.2	106.0	13.60	5.67
	81.9	1.55	1.26	7.77	16.2	110.8	14.49	5.71
	101.8	2.25	1.89	8.44	22.4	107.0	14.94	5.82
	155.0	2.33	1.78	8.00	22.31	109.8	14.18	5.69
	670.0	5.06	2.68	8.52	31.4	108.2	14.46	5.76

* Each experiment number represents measurements made on a single collection of blood.

† Sodium bicarbonate added to the blood.

‡ Lactic acid added to the blood.

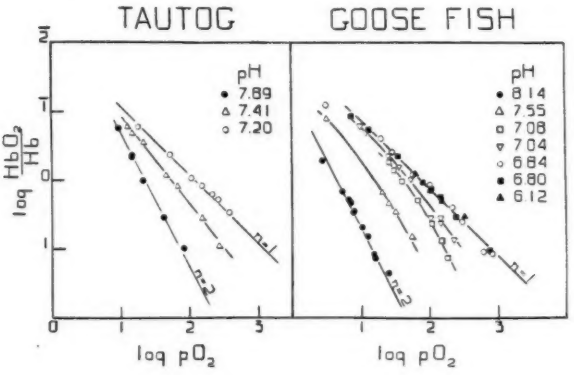

FIG. 3. For explanation of this figure, see text.

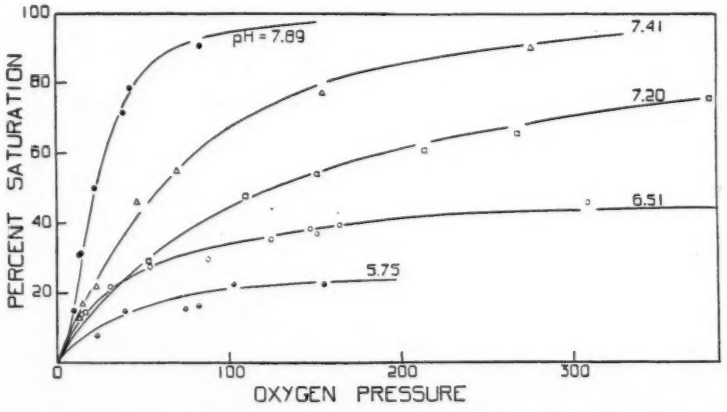

FIG. 4. Oxygen dissociation curves at constant pH of the blood of the tautog. The curves have been drawn according to equation (3) using the following constants:

pH	α_1	α_2	α_0	K_1	$K_2 \times 10^2$	$1/K_1$	$1/K_2$
7.89	0	1.0	0		.19		23[2]
7.41	.5	.5	0	.0286	.0156	35	80[2]
7.20	1.0	0	0	.008		125	
6.51	.5	0	.5	.022		45	
5.75	.3	0	.7	.022		45	

more acid curves for tautog blood are rectangular hyperbolæ and K_1 is the same at pH 6.5 and 6.1 although the oxygen capacity is

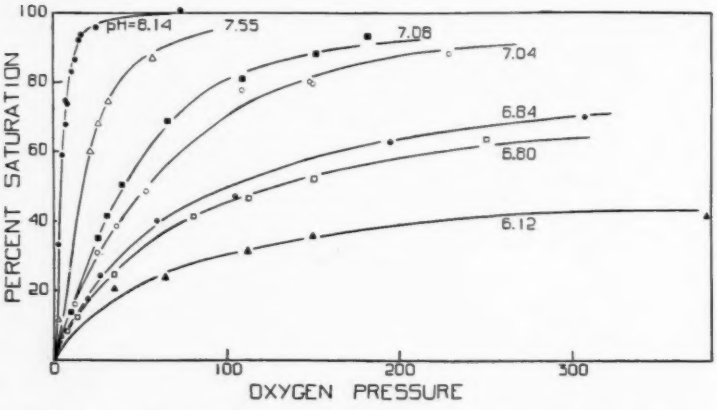

FIG. 5. Oxygen dissociation curves of the blood of the goosefish at constant pH. The curves have been drawn according to equation (3) using the following constants:

pH	α_1	α_2	α_0	K_1	K_2	$1/K_1$	$1/K_2$
8.14	0	1.0	0		.05		4.47^2
7.55	.25	.75	0	.067	.0011	15	30^2
7.07	.45	.55	0	.04	.0004	25	50^2
7.04	.50	.50	0	.04	.000158	25	80^2
6.84	.88	0	.12	.0131		76	
6.80	.80	0	.20	.0131		76	
6.12	.54	0	.46	.0131		76	

diminished. The same is true of the goosefish blood at pH 6.84, 6.80, and 6.12.

The change in the position of the curves with pH is very marked in these bloods as well as in that of the toadfish. In the tautog there is an additional phenomenon. The oxygen dissociation constant for the curve at pH 7.2 is considerably smaller than that for the curves at lower pH values. That is, at first the curves are shifted to the right with increasing acidity but at higher acid concentrations there is a reversal of the effect. A similar reversal of the Bohr effect was found by Rona and Ylppö (1916) working on dog blood, by Ferry and Green (1929) for horse hemoglobin, and by Stedman and Stedman (1926) and Hogben (1926) for crustacean hemocyanin.

The most interesting single characteristic of these curves is the change in shape from a rectangular hyperbola to a sigmoid curve. Unlike the toadfish, in which one component behaves as though $n = 2$ and one component as though $n = 1$ at all pH values, the change in shape must be due to a change in behavior of the component groups.

Thus, whatever the theory used as a basis for the description of the curves, it may be observed that acidity has a threefold effect on the blood; first, a change in the value of the oxygen dissociation constant, second, a decrease in oxygen capacity, third, a change in the shape of the curves describing the equilibrium with oxygen.

Theoretical Deductions Concerning the Loss in Oxygen Capacity in Acid Solutions

Henderson (1920) has shown that the effect of acid on the combining power of hemoglobin with oxygen could be described in terms of four mass law expressions; one for the combining power of oxygen with acid hemoglobin, HHb, one for oxygen with salt hemoglobin, BHb, and one each for the acid dissociation of oxygenated and reduced hemoglobin, $HHbO_2$ and HHb respectively. Thus the ease with which oxygen combines with hemoglobin depends upon the dissociation of the latter as an acid and oxyhemoglobin is a stronger acid than reduced hemoglobin.

The situation becomes rather complicated when more than one molecule of oxygen is considered to combine with one molecule of hemoglobin. By making certain assumptions concerning the interdependence of the four prosthetic groups, it is possible to derive an expression predicting the relation between oxygen capacity and hydrogen ion concentration exhibited by the data in Fig. 1. By making a few assumptions it is possible to explain this phenomenon of reversible loss of oxygen capacity in relatively simple chemical terms. The fundamental conception is that the oxygen combination is intimately dependent upon the acid dissociation of the hemoglobin as in mammalian blood, but that the effect is so exaggerated that oxygen, at ordinary oxygen tensions, will not combine with the hemoglobin unless the latter is in the ionized state. This is, of course, not the only possible theory but it is set forth here as a simple interpretation of the mechanism of a phenomenon apparently very different from our usual conception of the equilibrium between oxygen and hemoglobin.

Let it be assumed:

1. That each hemoglobin molecule contains four groups capable of

combining with oxygen and that each of these groups is associated with the dissociation of two hydrogen ions.[2]

2. Assume further that only the ionized prosthetic groups, that is, the groups from which hydrogen ions have been dissociated, can combine with oxygen.[3] Then in the presence of oxygen sufficient to combine with all the ionized hemoglobin the number of forms present is reduced to H_8Hb, $H_6HbO_2^-$, $H_4Hb(O_2)_2^{=}$, $H_2Hb(O_2)_3^{\equiv}$, and $Hb(O_2)_4^{\equiv=}$.

3. Assume finally that the first two pairs of H ions dissociate from the hemoglobin separately but the last two pairs dissociate in a single step; then there never is present an appreciable amount of the form which can combine with three molecules of oxygen. This is in accordance with the usage of equation (3) as applied to the oxygen dissociation curves of toadfish blood.

The equilibrium reactions for the acid and oxygen dissociation of the hemoglobin molecule according to these restrictions are reduced to

$$H_8Hb = H_6Hb^- + 2H^+ \qquad H_6Hb^- + O_2 = H_6Hb(O_2)^-$$

$$H_6Hb(O_2)^- = H_4HbO_2^{--} + 2H^+ \qquad H_4HbO_2^{--} + O_2 = H_4Hb(O_2)_2^{--}$$

$$H_4Hb(O_2)_2^{--} = Hb(O_2)_2^{\equiv=} + 4H^+ \qquad Hb(O_2)_2^{\equiv=} + 2O_2 = Hb(O_2)_4^{\equiv=}$$

If the oxygen tension is sufficiently high to convert practically all of the hemoglobin from which the hydrogen has been dissociated into the corresponding oxygenated form, the intermediate product of each pair of equations is never present in appreciable amount and the following equilibrium reactions and mass law expressions may be assumed to describe the limiting conditions:

$$H_8Hb = H_6HbO_2^- + 2H^+ \qquad \frac{[H_6HbO_2^-]}{[H_8Hb]} = \frac{K_1}{[H^+]^2}$$

$$H_6HbO_2^- = H_4Hb(O_2)_2^- + 2H^+ \qquad \frac{[H_4Hb(O_2)_2^{--}]}{[H_6HbO_2^-]} = \frac{K_2}{[H^+]^2}$$

$$H_4Hb(O_2)_2^{=} = Hb(O_2)_4^{\equiv=} + 4H^+ \qquad \frac{[Hb(O_2)_4^{\equiv=}]}{[H_4Hb(O_2)_2^{--}]} = \frac{K_3K_4}{[H^+]^4}$$

[2] The assumption that the combination of each oxygen molecule is accompanied by the dissociation of two hydrogen ions is in accordance with the finding of Ferry and co-workers (1929) that K_1 in equation (2), as applied to horse hemoglobin and to sheep blood, varies with the square of the hydrogen ion concentration, and with the observation of Redfield and Ingalls (1932) that the oxygen dissociation constant for certain hemocyanins also varies with the square of the hydrogen ion concentration.

[3] This also has a parallel in the hemocyanins, where the addition of acid produces a colorless compound incapable of combining with oxygen. See the end of this discussion.

The relative concentrations of the different forms present at any given pH may be readily calculated either by the logarithmic form usually employed in treating titration curves or in an algebraic form similar to the oxygen dissociation equations. Thus for the first equilibrium if

$$[H_6Hb] + [H_6HbO_2^-] = 1,$$

then

$$\frac{[H_6HbO_2^-]}{1 - [H_6HbO_2^-]} = \frac{K_1}{[H^+]^2} \text{ and } [H_6HbO_2^-] = \frac{K_1}{[H^+]^2 + K_1}$$

and similarly for the other expressions,

$$[H_4Hb(O_2)_2^=] = \frac{K_2}{[H^+]^2 + K_2}, \text{ and } [Hb(O_2)_4^{==}] = \frac{K_3K_4}{[H^+]^4 + K_3K_4}.$$

The total amount of oxygen bound at any given pH is the sum of all of the oxygenated forms.

$$H_6HbO_2^- + H_4Hb(O_2)_2^= + Hb(O_2)_4^{==}$$

Since only one quarter of the total oxygen bound can be in the form $H_6HbO_2^-$, the concentration as derived from the equilibrium expression must be multiplied by 0.25 as must also the concentration of $H_4Hb(O_2)_2^=$, while the concentration of $Hb(O_2)_4^{==}$ must be multiplied by 0.50. Substituting these values and the mass law expressions for the various oxygenated forms the total oxygen combined, Z, becomes

$$Z = .25\frac{K_1}{[H^+]^2 + K_1} + .25\frac{K_2}{[H^+]^2 + K_2} + .50\frac{K_3K_4}{[H^+]^4 + K_3K_4}.$$

In applying these equations to the data in Fig. 1, it must be kept in mind that the shape of the oxygen dissociation curves indicates that the hemoglobin would combine with slightly more oxygen if the oxygen tension were sufficiently increased. The theoretical oxygen capacity may be calculated in the more acid range because the curves are simple rectangular hyperbolæ and in all three bloods the oxygen dissociation constant has the same value in the most acid curves. If it be assumed that the constant is the same at intervening and lower pH values, knowing the oxygen tension and the concentration of oxygenated hemoglobin, it is possible to calculate the limiting amount of oxygen with which the hemoglobin can combine at these reactions. These values have been calculated using the data given in Fig. 1 for the goosefish below pH 6.8 where $K_1 = .013$, for the tautog below pH 6.55 where $K_1 = .022$, and for the toadfish below 6.5 where

$K_1 = .047$. At higher pH values the capacities have been calculated somewhat less accurately. The points between pH 6.55 and 6.96 for the tautog have been calculated using the K_1 values at both these reactions, then averaging the results which are then $\pm$ 2 per cent of

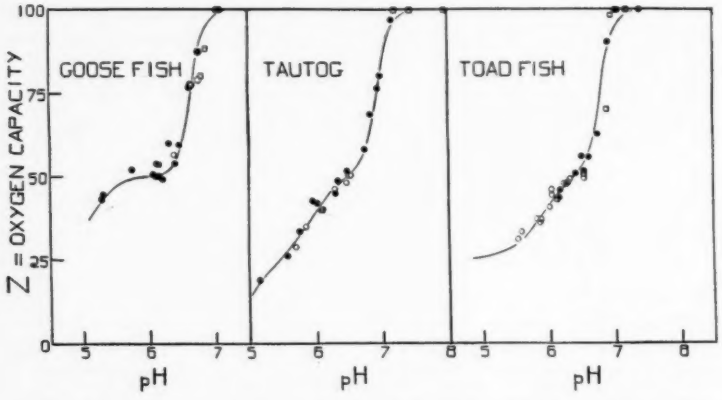

FIG. 6. The oxygen capacity of certain fish bloods. The points are calculated as described in the text:

- ● from the blood equilibrated with oxygen as given in Fig. 1.
- ○ from the blood equilibrated with air as given in Fig. 1.
- □ from the oxygen dissociation curves in Figs. 2, 4, and 5.

The curves are drawn according to equation (4).

the correct value. At oxygen tensions of 700 mm. the first half of the toadfish hemoglobin is practically saturated and the capacity between pH 6.55 and 7.0 has been calculated according to equation (3) using K_2 as 3.33×10^{-6}. All of the calculated points are represented in Fig. 6.

The curves in this figure are drawn according to equation (4) where $\sqrt{K_2}$ and $\sqrt[4]{K_3K_4}$ are for the goosefish $10^{-5.1}$ and $10^{-6.65}$ respectively; for the tautog, $10^{-5.93}$ and $10^{-6.95}$ respectively, and for the toadfish $10^{-5.85}$ and $10^{-6.8}$ respectively. $\sqrt{K_1}$ is approximately 10^{-5} for the tautog but is too large to be determined for the other two fishes.

The applicability of this type of equation to the oxygen capacity supports the interpretation of the oxygen dissociation curve as developed in the case of the toadfish. The equation is also adequate to describe the relation observed in tautog and goosefish blood, although the assumptions used in deriving curves to describe the oxygen equilibrium are somewhat different from those employed for toadfish blood. This difference suggests that in some cases there may be an

interdependence of the acid dissociation of the prosthetic groups which is not paralleled by an interdependence in the combination of oxygen.

The most important implication of the curves in Fig. 6 is the same as that derived from the undulatory character of the oxygen dissociation curves of the toadfish, namely, hemoglobin contains prosthetic groups differing in their behavior with respect to hydrogen dissociation and oxygen combination.

The evidence for this is the inflection of the tautog and toadfish curves at 50 per cent saturation and the actual break in the goosefish curve at the same point. Thus the effect of the addition of acid, as originally suggested (Root, 1931), may be interpreted as the inactivation of prosthetic groups on the hemoglobin molecule. This inactivation is probably due to the undissociated character of the hemoglobin as an acid, oxygen combining with only the ionized form of hemoglobin.

A similar inactivation of respiratory pigment in the presence of acid has been described by Redfield, Mason, and Ingalls (1932). The hemocyanin of *Limulus polyphemus* reacts with hydrochloric acid to form a component that is colorless and does not react with dissociable oxygen. The colorless component may be separated from a partially acidified hemocyanin solution by the addition of a strong solution of sodium chloride. When such a separation has been effected, analysis of the nitrogen in the filtrate indicates that its protein content has been diminished to just such an extent as the hemocyanin has been decolorized. Here, also, the combination of each oxygen molecule appears to be dependent upon the dissociation of two hydrogens, for the oxygen dissociation curves are rectangular hyperbolæ and the equilibrium between hydrochloric acid, hemocyanin, and the resulting colorless component may be described by an equation in which the hemocyanin is behaving as a divalent acid or base.

We wish to express our thanks to Professor Alfred C. Redfield for his generous interest and direction throughout the course of this investigation.

Summary

1. The oxygen dissociation curves of the blood of certain marine teleosts, the toadfish, the goosefish, and the tautog, have been studied.

2. The oxygen dissociation curves for the toadfish are undulatory in character with two areas concave to the abscissa. The second inflection begins at approximately one half saturation.

3. The oxygen dissociation curves for the goosefish and the tautog change shape with change in pH. At alkaline reactions the curves are sigmoid whereas in acid solutions they are rectangular hyperbolæ.

4. The form of the oxygen dissociation curves at constant pH can be described by Hill's equation only under a limited number of circumstances. The curves for the toadfish may be described in terms of equations derived from the assumption that each molecule of hemoglobin combines with four molecules of oxygen.

5. The position of the dissociation curves of all of these fish bloods is markedly affected by pH, and, in addition, with increasing acidity the oxygen capacity is reduced.

6. The manner in which the addition of acid lowers the oxygen capacity may be deduced from the assumption that oxygenation is dependent upon the dissociation of hemoglobin as an acid.

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OBSERVATIONS UPON STENOSTOMUM ŒSOPHAGIUM

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The genus *Stenostomum* has been studied frequently since its first species was described by Ant. Dugès in 1828 as *Derostoma leucops*. There is considerable confusion in the records concerning the anatomy and life-history of the species of this genus. Our observations have to do with certain anatomical details and phenomena dealing with sexual propagation.

MATERIAL AND METHODS

The species *S. œsophagium* was described by Kepner and Carter in 1930, their description being based upon the first of the two clones employed by us. This first clone arose from an individual collected in September, 1929, and was maintained, in numerous lines, until July, 1930, when it died during the intense heat of the season. The second clone was established from a collection made in the last week of August, 1930. At the present writing, December, 1932, this clone is still running. Cultures have been maintained in wheat infusions,[1] little attention being paid to the hydrogen ion concentration. The range of pH for satisfactory cultures is from 5.6–7.6. Our most thrifty specimens have appeared in culture media that were on the acid side as low as pH 6.3.

The best material for sectioning was obtained by using hot (about 50° C.) Zenker's fluid. Iron hæmatoxylin, with eosin as a counterstain, was employed for the most part.

GENERAL ANATOMY

The spindle-shaped body of this species measures about 1.5 mm. in length when no obvious fission plane is present. A specimen that has experienced inanition is colorless. A well-fed individual, on the other hand, has a yellow-brown tint due to the presence of absorbed food within the enteric epithelium. There are three external apertures: a ventral sub-terminal mouth; a ventral nephropore near the middle of the caudal region; and a dorsal, male gonopore that lies over the mouth. The body is covered with cilia.

[1] Four grains of wheat were boiled in 100 cc. of spring water and inoculated with bacteria, rotifers, and protozoa.

The epidermis bears numerous rhabdites (Fig. 1*B*, *r*) which lie in slender, parallel groups at the bases of the epidermal cells and expand distally to become almost uniformly distributed beneath the outer surface of the epidermis. In the fixed condition, the epidermis, from which the rhabdites have been discharged, shows deeply-staining chromatic lines (Fig. 2, *l*) that converge in each cell to form a common stem lying by the nucleus. However, these common stems show no morphological connection with the nuclei.

The alimentary canal consists of a mouth, pharynx, œsophagus, and enteron. These are all lined with a ciliated epithelium. The wall of the pharynx (Figs. 1 and 3, *ph*), is more muscular than that of the œsophagus. Furthermore, many unicellular glands (Fig. 3, *pg*) are loosely applied to the outer surface of the pharynx. These glands open into the lumen of the pharynx at its anterior end directly behind a small, dorsally directed diverticulum (Fig. 3, *d*). None of these glands crowd back over the œsophagus (Figs. 1 and 3, *œ*). Food-objects are delivered by the pharynx to the œsophagus, where they are held for a time before they are passed on to the enteron. A sphincter guards the passageway between these two regions of the alimentary tract. The enteron is an oval sac with a lining of ciliated endodermal cells. This epithelium (Figs. 1 and 3, *en*) in the living animal, under the usual vegetative conditions, presents a densely granular appearance. It is made up of tall, ciliated columnar cells (Fig. 4, *enc*), which may leave the epithelium and pass out into the pseudocœle. Figure 4 shows a cell

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Explanation of Figures, Plate I

FIG. 1. *A*. Dorsal aspect of a living specimen. *en*, enteron; *mgs*, male genital system; *oe*, œsophagus; *ov*, *ov'*, *ov''*, ovaries; *ph*, pharynx; *pn*, protonephridium. × 80. After Kepner and Carter (1930).

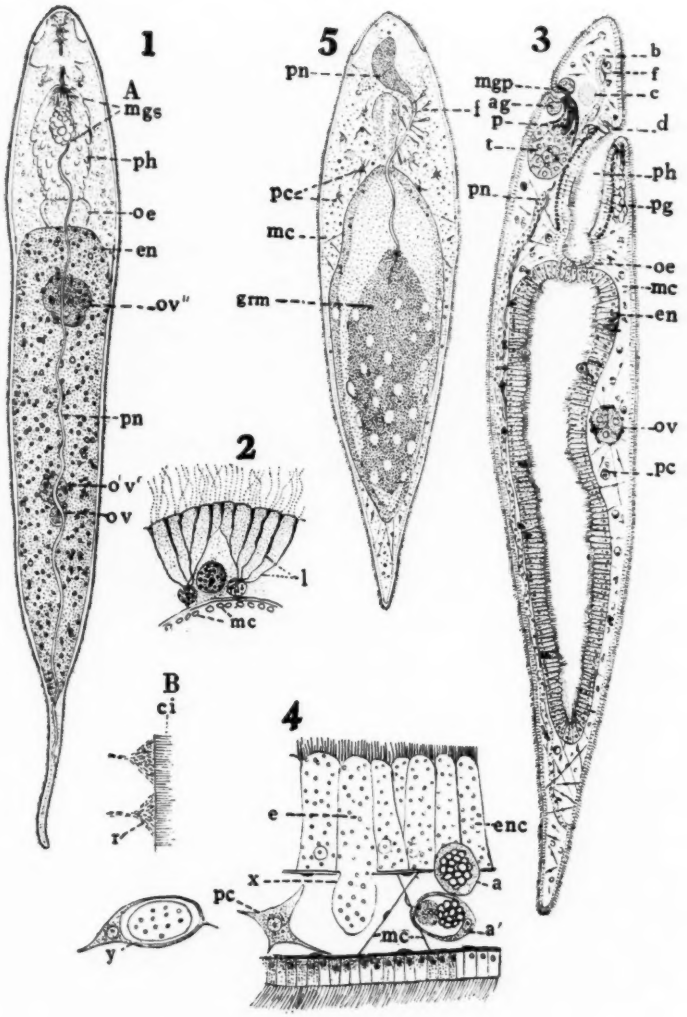
B. Region of living epidermis showing cilia (*ci*), and rhabdites (*r*).

FIG. 3. Sagittal section involving median plane. *ag*, accessory gland of male genital atrium; *b*, flexed part of protonephridium; *c*, neural commissure; *d*, dorsal diverticulum of pharynx; *f*, capillary region of protonephridium; *en*, enteron; *mgp*, male genital pore; *oe*, œsophagus; *ov*, ovary; *p*, penis; *pc*, phagocyte; *pg*, glands of pharynx; *ph*, pharynx; *pn*, main stem of protonephridium; *t*, testis. × 80.

FIG. 4. A region involving epidermis, pseudocœle, and endoderm of a living specimen as seen under water immersion objective. *a*, a wandering cell leaving epithelium of enteron, loaded with small bodies that it had taken up within the endoderm; *a'*, a similar cell that lies within the pseudocœle; *e*, endodermal cell leaving the epithelium, later its projecting end was sheared off; *enc*, endermal cell; *mc*, muscle cells; *pc*, phagocyte, which at *y* had ingested the fragment of endodermal cell that had been broken from *x*.

FIG. 5. Dorsal aspect of a specimen that had but recently deposited an egg. *f*, flame-cell; *grm*, granular material within the very thin-walled enteron; *mc*, muscle cells; *pc*, phagocytes; *pn*, region of protonephridium distended by granular inclusions. × 96.

PLATE I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that was leaving the epithelium while being observed under a water immersion objective. The shearing movements of the body-wall, however, tore the projecting part from the cell. This fragment of endodermal cell was soon ingested by the phagocyte (*pc*), which lay near, with the result shown in Fig. 4 at *y*. Between the ciliated cells of the enteron's epithelium other small cells may be found. We have seen these small cells appropriate refractive bodies, as they lay within the enteric epithelium, and then migrate from the wall of the enteron into the pseudocœle as shown in Fig. 4 at *a* and *a'*. This is taken up in greater detail by J. S. Carter (1933).

There are two bi-lobed cephalic ganglia, lying anterior to the mouth, with their larger anterior lobes in contact with the ciliated pits. An "eye-spot" is attached to each posterior lobe. The cephalic ganglia are connected by a transverse commissure (Fig. 3, *c*).

The protonephridium (Figs. 1 and 3, *pn*) is a conspicuous organ of the pseudocœle. This protonephridium consists of two regions, (1) a main stem and (2) a capillary-like portion. The main stem is the more conspicuous because of both its relative size and density. This entire organ lies in the mid-line, dorsal to the organs of the pseudocœle, except where it passes beneath the neural commissure. Anterior to this commissure, the protonephridium is bent upon itself. This deflection (Fig. 3, *b*) marks the boundary between the two regions of the protonephridium. The main stem has a thick cytoplasmic wall within which lie many nuclei, but in which no cell boundaries have been seen either in fixed or living material. The deflected part of the protonephridium appears to be a thin-walled syncytium. This capillary-like, posteriorly deflected region lies ventral to the main stem and is closely applied to it at irregular intervals. It receives scattered branches that have a similar structure. It is into this capillary-like portion of the protonephridium that the flame-cells (Figs. 3 and 5, *f*) empty. The anatomy of the

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Explanation of Figures, Plate II

FIG. 6. A phagocyte lying over a peripheral muscle cell (*m*). × 440.

FIG. 7. A median sagittal section of male reproductive system. *ag*, accessory gland; *mgp*, male gonopore; *p*, penis; *sp*, spermatids; *sv*, seminal vesicle; *t*, testis. × 416.

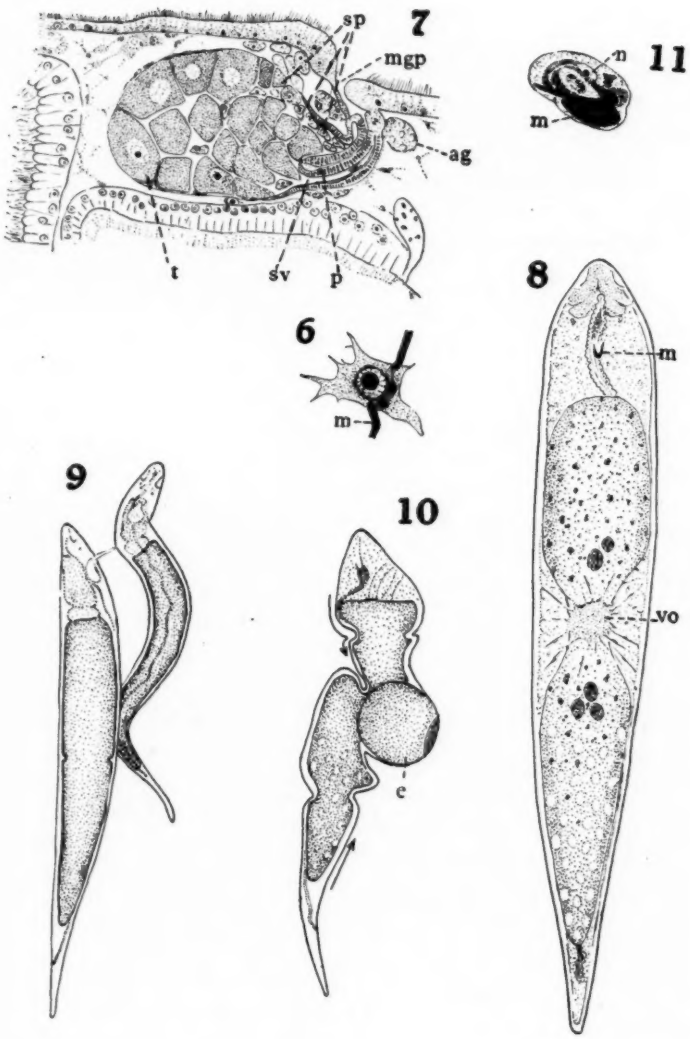
FIG. 8. Ventral aspect of a specimen that had but recently deposited an egg. *m*, mouth; *vo*, mesenchymal structure left in region in which egg had lain. × 80.

FIG. 9. A copulating pair in which the larger male is anchored to the anterior right side of the smaller one by the former's slender, colorless penis.

FIG. 10. Lateral aspect of mature female. Note reduced anterior or cephalic region. *e*, egg emerging as peristaltic waves travel towards it (indicated by arrows).

FIG. 11. A phagocyte that has ingested a muscle cell. *m*, muscle cell; *n*, nucleus of phagocyte. × 440.

PLATE II



protonephridium of this *Stenostomum* is, therefore, similar to that described for *S. leucops* by Westblad (1923). Granular material has been observed frequently within the ciliated lumen of the thick-walled region but never within the lumen of the thin-walled region. This observation supports Reisinger's (1922, 1923) statement that the thick-walled region has an excretory function, while the thin-walled region has a filtering function.

Slender, non-striated, muscle-cells are associated with the body-wall, the walls of the enteron, œsophagus, and pharynx; while others (Figs. 3 and 5, *mc*) radiate from the alimentary canal to the body-wall.

Several types of leucocytes (Figs. 3, 4, and 5, *pc*) drift more or less passively within the pseudocœlomic fluid or crawl, amœba-like, over surfaces of muscle cells, between epithelial cells or to and into any other regions of the body. Figures 6 and 11 show two of these leucocytes.

The gonads also lie within the pseudocœle.

Reproductive Organs

Vejdovsky (1880) was the first to have seen the male reproductive system, but he failed to recognize it as such and designated it the "ovale Drüse." Von Graff (1882) described only the female organs, while Sillimann (1885) saw the testis and expressed the opinion that the "ovale Drüse" of Vejdovsky was a sex organ. Voigt (1894) recognized both male and female gonads; while Keller (1894) gives a description of both the testis and the ovary. Landsberg (1887) records, "Hoden sah ich in derselben Kette, in der sich auch das Ovarium befand." Voigt (1894) likewise observed that *Stenostomum* was monœcious, while Sekera (1903) confirmed these observations. Von Graff (1913) states that "Die Geschlechtsorgane sind nur teilweise bekannt" (s. 18). Meixner (1924) records, "Hiezu sei vorläufig bemerkt, dass die dorsale Lage der männlichen Geschlechtsöffnung im Vorderkörper nur für etwa vier *Stenostomum-Arten* erwiesen ist" (s. 124). Sonneborn (1930) says, "Little is known concerning sexual reproduction in these organisms" (p. 59). Sabussow (1897) gave the most nearly complete description of the male reproductive system. He recognized five parts to this system in *S. leucops:* (1) a single testis; (2) a spheroidal seminal vesicle; (3) a tubular penis, lacking chitin, measuring "13 μ long and 11 μ breit"; (4) a penis-sheath; and (5) a small "antrum masculinum."

The male reproductive system of *S. œsophagium* corresponds to the description of that of *S. leucops* as given above by Sabussow (1897), except for the short tubular penis depicted and described by this author. We find that lying within the terminal duct, or neck, of the male organ

is a highly muscular, tubular penis (Figs. 3 and 7, *p*). This occupies a position similar to that of the slime (*Schl* of Sabussow's Taf. 5, Fig. 1) which this author depicts and describes. We are therefore inclined to consider that Sabussow had mistaken the real penis of *S. leucops* for slime. The penis is an inverted tube capable of being everted. Its base, in the resting condition, gives the suggestion of a short tubular penis with a shallow penis-sheath. In reality it is clearly seen that in *S. œsophagium* there is a long tubular penis and that the penis-sheath shown in Sabussow's Fig. 1 is but a part of the genital antrum, the penis being housed, as a matter of fact, within the muscular seminal vesicle (Fig. 7, *sv*). In our Fig. 7 some spermatids are shown lying dorsal to the penis at *sp*.

Individuals which arise from eggs in the laboratory may become sexually mature, as males, in about three weeks. For example, an individual that emerged from the egg on December 16 was found to be sexually mature, as a male, on January 6. Sexually mature males may propagate asexually for many generations before female gonads appear.

There are no gonoducts associated with the female gonads. When oögonia are first seen in the living specimen, they lie beneath the ventral mid-line of the enteron within the pseudocœle. The growing primary oöcytes become enclosed in a loose, muscular tunic that is, in turn, supported by radiating non-striated myocytes. This rather indefinite aggregate of cells constitutes the ovary (Fig. 3, *ov*). We are not, as yet, in a position to state whether the oögonia are of mesenchymal or of endodermal origin. We are inelined to believe that they are diapeditic cells of the enteric epithelium. This subject will be taken up by one of us later.

Sekera (1903) observed that during the development of the "egg" four cells appear, one of which may function as the gamete and the remaining three may be used as yolk material. Our observations show that by the time the developing ovary becomes discernible in the living specimen, it is composed of two cells (Fig. 1, *ov*). After it has grown but a little more, the ovary contains four equal cells (Fig. 1, *ov'* and *ov''*). The gonad suggests the appearance of an embryo of four blastomeres. These cells, however, are not blastomeres. Despite the fact that there are thus always four cells in an advanced ovary, no ovary gives rise to more than one zygote. Only one of the four cells becomes the functional gamete, the other three having disappeared before egg-deposition. Both Sekera (1903) and Van Cleave (1929) indicate that at least the first cleavage of the zygote occurs before egg-deposition. We, however, are not in a position to confirm this.

A single ovary usually develops, but we have observed as many as

seven ovaries develop and reach maturity in a given worm. The ovary usually lies in the anterior zoöid in specimens that present both a fission-plane and an ovary. However, specimens have been found frequently that displayed a well-defined fission-plane with an ovary in each zoöid. In these examples the anterior ovary was usually the more advanced (larger).

Copulation

There being no female gonoducts, copulation must be peculiar. We have observed two cases of copulation.

On one occasion, while a specimen that showed no discernible ovary was feeding, a second individual in a similar sexual condition (lacking a discernible ovary, but possessing a complete male genital system) approached the mass of food in such a way that its left anterior region lay near, but not in contact with the anterior end of the first animal. The penis of the first animal was everted with a high velocity so that its end pierced or adhered to the epidermis of the second male specimen (Fig. 9). Each animal, thereupon, made efforts to free itself as the two were held together by the translucent penis of the first animal. Their efforts eventually resulted in separation. The penis was then quickly drawn back into the seminal vesicle.

On another occasion we saw a similar series of phenomena differing only in that the point of contact of the penis with the second animal's body was at a different level from that involved in our first observation.

Voigt (1894) says, "Die männlichen Geschlechtsprodukte reifen erheblich früher als die weiblichen, so dass eine Selbstbefruchtung ausgeschlossen erscheint" (s. 747). Keller (1894) also says, "Die männliche Geschlechtsreife tritt erheblich früher auf, als die weibliche. Selbstbefruchtung ist somit nicht wohl anzunehmen" (s. 398). Sekera (1906) says, however, ". . . kommt es nach der Ausbildung der Dottermasse in den Keimzellen zur Selbstbefruchtung in der Weise, dass die Hodenfollikel platzen und reife Spermatozoen in der Leibeshöhle herumschwärmen, bis sie in die eine oder andre Keimzelle der einfachen Ovarien, welche nur aus 4 Keimzellen bestehen, hineindringen und zur Ausbildung eines Eichens mit dicker Eischale beitragen" (s. 142).

Our observations of copulation support the opinion of the first two authors. Furthermore, since copulation took place between individuals, each of which had no discernible ovary, it is suggested that the presence of foreign spermatozoa may be necessary for the development of latent oögonia. This suggestion is strengthened by the fact that we have no definite examples of isolated individuals becoming sexually mature as females; but our evidence in this connection is not sufficiently extensive to be convincing.

Egg-Deposition

The absence of female gonoducts has long attracted the attention of investigators. Voigt (1894) states, "Nach der Eiablage sterben die Tiere nicht ab, sondern sie fangen schon vor Beendigung derselben an, sich wieder durch Teilung fortzupflanzen" (s. 747). Von Graff (1913), Sekera (1926), and Van Cleave (1929) record that the egg is discharged by rupturing the epidermis, resulting in the death of the parent. Carter (1930) observed that while egg-deposition brings on a critical period, it is not necessarily fatal to the mother.

We have observed the details of egg-deposition. On January 25, 1931, we discovered a specimen that had an ovary surrounded by a translucent shell. The ovary's presence caused a conspicuous protuberance near the middle of the animal. The region in the immediate vicinity of the ovary displayed great and sustained muscular contraction. The regions anterior and posterior to the ovary presented peristalsis, the waves of which traveled towards the large ovary (Fig. 10). While we took turns watching this specimen (which was quiet, except for the peristalsis), one of us saw the epidermis open and slip back over the egg and close behind it as though the epidermis were an elastic membrane with a small pore through which the egg had passed by distending the pore. The pore closed after the egg's passage had been effected, leaving no wound in the body-wall. Neither cells nor plasma could be seen to have escaped with the egg. Within the pseudocœle lay the vestige of the muscular tunic of the ovary (Fig. 8, *vo*). We have made other similar observations of egg-deposition.

The period of incubation of the eggs, under laboratory conditions, varies greatly. Van Cleave (1929) obtained two incubation periods of twenty-five days each. We have had a period as short as fifteen days and one that extended well over a month. However, few eggs deposited under laboratory conditions developed.

The Influence of the Female Gonads upon the Parent

The ovary has a marked influence, both upon the life of the clone and that of the individual, for its presence influences both the fission-rate and the anatomy of the individuals. Van Cleave (1929) records that fission is inhibited by the ovary. Landsberg (1887), on the other hand, observed chains of zoöids in which the zoöids had female gonads. We have recorded frequent cases of fission in which the ovary lay in the anterior zoöid; so we may say that when an ovary appears anterior to an incipient fission-plane, this fission-plane is not inhibited in all cases; but a second fission-plane does not appear.

When, however, an ovary develops posterior to the fission-plane the usual events may not occur in the plane of division. The following is an example of such influence of a posterior ovary. January 24, 1931, we discovered an animal that was unusually long and had a fission-plane. Ordinarily a specimen with an advanced fission-plane measures about 3 to 4 mm. in length. This specimen, however, was 7 mm. long. The posterior zoöid had a conspicuous ovary. The anterior zoöid had a minute ovary that could be seen only with high magnification. The fission-plane's constriction indicated that it was an advanced one, but there were rudiments neither of ciliated pits, pharynx, cephalic ganglia, nor testis posterior to this plane of constriction. There was a small testes in the anterior zoöid. The enteron of the posterior zoöid housed within its wall refractive, oval bodies. On January 25 the specimen was indifferent to food. The posterior ovary had grown while the anterior one had not. On January 26 the posterior ovary had discharged its egg, the zoöid showing the depression within which the egg had been lying. The anterior ovary had not grown, but by January 27 the anterior ovary had enlarged. The posterior zoöid still showed the displacement of tissues that had been caused by the presence of the ovary, but no cephalic organs had developed. On January 28, the specimen had divided. At this time, the posterior zoöid lacked all cephalic organs. However, two weeks later this posterior zoöid had developed a pharynx, ciliated pits, and cephalic ganglia. This is but one of frequent similar examples that we have seen. Thus it appears that when an ovary is more advanced in the posterior zoöid, the formation of organs or differentiation of tissues in the region of the fission-plane is inhibited.

Sekera (1903) observed " Bald darauf beginnt auch der Pharynx zu degenerieren und das Individuum nimmt keine Nahrung auf, indem es durchsichtig und verkümmert zu werden pflegt " (s. 543).

We have found that the presence of a developing ovary makes an increased demand for material that must be supplied at the expense of the substance of the body as a whole. The leucocytes of the mesenchyme increase in number in the vicinity of the pharynx as the ovary grows. These become phagocytic and attack the radiating muscles of the pharynx and ingest them (Fig. 11). This phagocytosis eventually greatly reduces the size of the pharynx and œsophagus. The testis completely disappears. The main stem or thick-walled region of the protonephridium is resorbed at irregular levels, sometimes leaving only an enlarged sack-like region (Fig. 5, *pn*) as a vestige of the main stem, and the capillary region bearing flame-cells (Fig. 5, *f*). This vestige becomes greatly distended and contains a dense mass of minutely crystalline material (Fig. 5, *pn*). With advanced phagocytosis, the wall of the

enteron becomes quite thin and a mass of granular material (Fig. 5, *grm*) appears within its lumen. This material resembles that found within the distended anterior region of the main stem of the protonephridium at this time. It may be that with the reduction of the pharynx excreted materials can no longer escape by it and thus accumulate within the enteron as they do within the main stem of the protonephridium when it has been cut off from the exterior. The resorption of the anterior organs may be much more extensive than is indicated in Fig. 5. Frequently all of the cephalic structures have been resorbed.

The Turbellaria are usually considered to represent a phase in the phylogeny of the Annelida. That we should see extensive dedifferentiation associated with egg-formation in *Stenostomum* has some significance when the phenomenon known as epitoky in the annelids is considered; for it lends support to the theory that Annelida may have arisen from Turbellaria.

Some experiments were made to test the hypothesis that the reduction of the cephalic organs was necessary in order that material may be supplied to the egg. Specimens were taken that showed incipient ovaries. From these the "heads" were removed. In each case the wound healed, but no new "head" was formed until after the egg was laid. This supports van Cleave's (1929) observation that reconstitution does not occur in female specimens. However, the loss of cephalic tissue that would ordinarily be resorbed did not inhibit the growth of the ovary. In all cases the ovary reached maturity and an egg was laid. The growth of the ovary is therefore not dependent upon the resorption of the cephalic organs. It is suggested that the degree of resorption of cephalic organs, that takes place when the ovary is growing, is correlated with the relative age of the "head" involved. This is a line of observation that one of us plans to follow.

The Appearance of Male Gonads Dependent upon Extrinsic Conditions

Sekera's (1903) suggestion that the appearance of sex is determined by extrinsic factors is supported by our observations. Concerning *Stenostoma leucops*, he says "Aus meinen vieljährigen Beobachtungen, die ich schon vom Jahr 1885 an fort führe, kann ich mir erlauben zu behaupten, dass ein jeder Tümpel nach seiner physischen Beschaffenheit eine bestimmte Zeitdauer hat, in welcher alle Exemplare von *Stenostomum leucops, unicolor,* etc. geschlechtlich sich entwickeln. Damit hängt es auch zusammen, dass wir in den nassen Sommerzeiten, wo sich viel Wasser in den Tümpeln anhäuft, mit dem oben erwähnten

ungünstigen Umstande für die Ausbildung der Geschlechtsorgane nicht zu rechnen haben" (s. 538).

Specimens have been taken from their natural habitat in May, June, July, August, and September during three years. We have found these to be sexually mature as males only during the last week of August and in September. The species appear to be carried through the winter by zygotes, for extensive collections made in early December and early March yielded no examples of this species.

In contrast to this we have maintained a clone that always presented sexually mature males and at irregular intervals sexually mature females through the year. In addition to this, we have raised sexually mature males from the egg within a period of a month.

It thus appears that certain extrinsic factors of the natural habitat arise in late August which determine the appearance of male gonads, while under laboratory conditions extrinsic factors are present which prompt the development of male gonads throughout the year. *S. œsophagium,* in being sexually mature as males under laboratory conditions, stands sharply in contrast with *S. tenuicauda.* Nuttycombe (1932) has maintained a clone of *S. tenuicauda* for over five years, during which time gonads have not appeared, although specimens collected from their natural habitat were sexually mature in the autumn.

Summary

The male gonad and gonopore lie dorsal to the anterior end of the pharynx. There is no female gonoduct.

The absence of a female gonoduct is correlated with a peculiar mode of copulation.

Egg-formation and egg-deposition bring about a crisis in the life of the individual which is not necessarily fatal. Extensive dedifferentiation and phagocytosis are associated with the development of the egg. After the egg has been laid, a new "head" is developed.

Development of male gonads, in this rhabdocœle, appears to be dependent upon extrinsic rather than intrinsic factors.

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THE RELATION BETWEEN RATE OF LOCOMOTION AND FORM IN AMŒBA PROTEUS[1]

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Mast and Prosser (1932, p. 336) maintain that the rate of locomotion in *Amœba proteus* is highest in monopodal specimens, lowest in multipodal specimens, and intermediate in bipodal specimens, and that ". . . selection of monopodal specimens greatly reduces the variability in rate," but they present no evidence in support of their conclusions. The experimental results presented in the following pages concern these conclusions. They were obtained as follows:

About 50 specimens of *Amœba proteus* were taken at random from a culture, washed[2] three times in redistilled water, and transferred to a Pyrex glass dish containing .001N sodium chloride solution.[3] The dish was then put into a constant temperature apparatus (Pitts, 1932) on the stage of the microscope and left for one hour for adjustment. A single specimen was now selected and projected with a camera lucida on black paper; then the position of the posterior end of the projected image was marked at intervals of one minute, and the outline of the image of the animal sketched at the intervening half minute. This was continued for seven minutes after which it was repeated with each of four other specimens differing in form, and with each of five similar specimens in a number of other dishes. This was continued until records were in hand for 60 specimens, 20 monopodal,[4] 20 irregular monopodal, and 20 multipodal specimens. All of these observations were made on specimens which had been in the sodium chloride solution from one hour to one hour and 45 minutes. The temperature during the observations

[1] This investigation was carried on under the direction of Professor S. O. Mast.

[2] The washing was performed by putting the amœbæ into watch glasses containing redistilled water, allowing them to settle and attach, then removing the water with a pipette, and finally pouring on more redistilled water.

[3] Kahlbaum's purest sodium chloride, accurately weighed and dissolved in water redistilled from a tandem Pyrex glass still (Mast, 1928).

[4] A monopodal amœba is defined as an elongated cylindrical animal, smooth in outline, which moves continually by the projection of a single pseudopod. Marine limax amœbæ of the type used by Pantin (1923) are regularly monopodal in form. *Amœba proteus* readily assumes the monopodal form if it is allowed to become stellate in redistilled water and then transferred to a dilute salt solution (Mast, 1928).

was $23 \pm 0.5°$. The results obtained are presented in Tables I and II and Fig. 1.

The results in Table I indicate that the rate is highest in monopodal forms and lowest in multipodal forms, and that the variation in rate of the different specimens is least in monopodal forms and greatest in a random sample, the coefficient of variation being 11.4 for the monopodal forms, 17.8 for the irregular monopodal forms, 27.9 for the multipodal forms, and 34.4 for the random sample. Thus it is evident that the variability of any given selected group is considerably less than that of a random sample, and that selection of strictly monopodal forms leads to least variation. The results obtained consequently support the conclusions of Mast and Prosser.

Schwitalla (1924) claims that locomotion in *Amœba proteus* is rhythmical, and he describes a two-fold rhythm—a rhythm of short duration consisting of rapid accelerations and retardations and a rhythm

TABLE I

Summary of statistics concerning the rate of locomotion of monopodal and multipodal amœbæ.

Form	Number of Specimens	Mean Apparent Rate	Probable Error	Standard Deviation	Coefficient of Variation
		mm. per minute	*per cent of mean*		
Monopodal	20	21.05±.35	1.65	2.30±.25	11.40±1.26
Irregular monopodal	20	19.72±.53	2.69	3.52±.37	17.85±1.96
Multipodal	20	9.44±.40	4.20	2.63±.28	27.86±3.20
Totals and averages	60	15.99±.48	2.99	3.49±.34	34.36±2.35

of long duration superimposed upon the short periods. He says (p. 490): "Only rarely will the same rate of locomotion be found to have been sustained for two successive minutes. Sometimes such an uniform rate may be maintained for two or three minutes, and in exceptional cases for five and seven minutes." And (p. 492): "In two successive minutes, a doubled rate, or a rate reduced by one-half occurs with comparative frequency."

Hahnert (1932) made a study of locomotion of monopodal specimens of *Amœba proteus* over a period of some twenty minutes and found the rate of locomotion to be surprisingly constant. He found in one specimen, for example, an average rate of 252.6 micra per minute with a standard deviation of only 13.6 micra per minute or 5.4 per cent. Similar results were obtained with nine other specimens tested. He

found no indication of rhythmic variation in rate of locomotion like that observed by Schwitalla, and consequently concludes that "the process of ascertaining the rate of locomotion is greatly simplified by using monopodal specimens."

TABLE II

Data Concerning the Rate of Locomotion of Monopodal and Multipodal Amœbæ

Designation of Individuals	1	2	3	4	5	6	7	8	
Monopodal Specimens									
1st minute	20.0	20.5	22.5	22.5	20.0	21.0	23.0	19.5	
2d minute	20.0	18.0	21.0	23.5	21.0	22.0	25.0	21.0	
3d minute	22.0	20.5	21.0	23.0	20.0	21.0	24.0	20.0	
4th minute	21.0	20.5	21.5	22.0	22.0	22.0	23.5	20.0	
5th minute	20.5	20.0	21.5	22.0	22.0	22.0	23.0	19.0	Total
6th minute	20.0	21.0	21.5	20.5	23.0	23.0	22.5	20.0	average
7th minute	20.5	20.5		22.5	23.0	23.0	24.5	21.0	rate
Average rate	20.57	20.14	21.50	22.79	21.43	22.00	23.64	20.71	21.35
Irregular Monopodal Specimens									
1st minute	21.0	10.5	19.5	25.0	24.0	18.5	19.0	23.5	
2d minute	20.0	19.5	18.5	25.0	19.5	15.0	19.0	18.0	
3d minute	16.5	15.0	19.0	18.5	21.5	18.0	16.5	22.0	
4th minute	10.5	9.0	14.5	21.0	21.5	15.0	18.5	23.0	
5th minute	12.5	18.5	16.0	23.5	24.0	16.5	16.0	22.5	
6th minute	18.0	27.5	17.5	25.5	20.0	21.5	21.0	19.0	
7th minute	20.0	12.0	18.0			23.5	22.0	20.0	
Average rate	16.93	16.00	17.57	23.08	21.75	18.29	18.86	21.14	19.20
Multipodal Specimens									
1st minute	12.0	10.0	11.0	6.5	6.5	10.0	9.5	10.5	
2d minute	14.0	11.5	14.0	10.5	7.5	9.0	9.0	16.0	
3d minute	11.0	10.5	7.5	7.0	8.5	13.0	10.5	4.0	
4th minute	8.5	9.0	7.5	8.0	8.5	13.0	16.0	10.5	
5th minute	6.5	5.0	10.0	9.5	8.5	9.5	17.5	11.5	
6th minute	3.5	5.5	9.0	9.5	7.0	8.0	16.5	14.5	
7th minute	3.0	8.0	14.0	7.5	4.0	10.0	17.5	12.0	
Average rate	8.36	8.50	10.43	8.36	7.21	10.36	13.79	11.21	9.77

By examining the rate of locomotion obtained during each of the seven-minute periods of observation of the individual specimens studied in this investigation, some insight is obtained as to the nature and reason for the rhythmical variation in rate which Schwitalla observed and which

Hahnert maintains is not evident in monopodal specimens. By referring to Fig. 3 of Schwitalla's paper it will be seen that there was great variation in the form of the amœbæ used, *i.e.* that some were nearly monopodal, some bipodal, and others multipodal.

It is evident from Table II of the present paper, in which the rates for successive minutes for eight specimens were taken at random from each of the three groups of forms studied, that the variation from minute to minute is much less for monopodal than for irregular monopodal

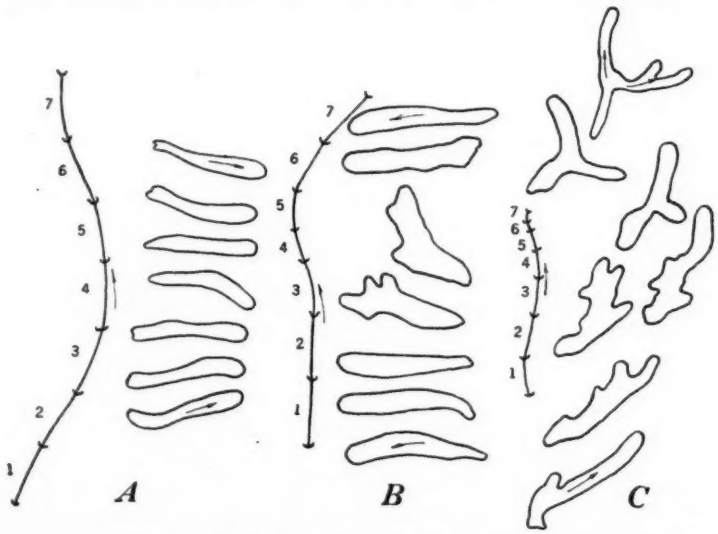

FIG. 1. Camera sketches illustrating the relation between form and rate of locomotion in *Amœba proteus;* curves, projected paths made by these specimens; arrows, direction of locomotion; cross lines on curves, position of the posterior end at minute intervals; outlines, form of the specimens at minute intervals during the time the paths were made.

Note the constant rate of locomotion attending the constant monopodal form (*A*); the decrease in rate as the form becomes irregular (*B*), fourth minute); the increase in rate as the monopodal form is assumed (*B*, seventh minute); and the decrease in rate as irregularity in form increases (*C*).

or multipodal specimens. For instance, by comparing specimen 1 of each of the three forms (Table II), it will be seen that the maximum variation within the seven-minute period for the monopodal specimen is 2 mm., or 9.7 per cent of the mean; for the irregular monopodal specimen 10.5 mm., or 62 per cent of the mean; and for the multipodal specimen 11.0 mm., or 131.6 per cent of the mean. This shows that the

greater the irregularity of the form of the amœba, the greater the irregularity of locomotion.

Figure 1 gives the records obtained concerning the rate of locomotion of these three specimens, and likewise the outline sketch of them at the intervening half minute. Figure 1*A* indicates clearly that as long as the amœba retains a strictly monopodal form it moves with a fairly uniform velocity. Figures 1*B* and 1*C* demonstrate that in variable specimens of irregular monopodal and multipodal form the marked increases in rate are for the most part due to the assumption of a monopodal or semi-monopodal form (Fig. 1*B*, seventh minute); and that the more irregular the form of the animal the slower the rate (Fig. 1*B*, fourth minute, and Fig. 1*C*). It consequently seems probable that the long rhythms which Schwitalla observed were due to change in form of the amœbæ. The short rhythms which he observed were probably due to periodic breaks in the plasmagel sheet, for with each break of this sheet (Mast, 1926) there is a forward spurt of locomotion (Mast and Prosser, 1932). It has, however, been demonstrated that selection of monopodal specimens reduces the variation between rates taken at different times on a single individual. Consequently, in experiments dealing with the effect of environmental factors on rate of amœboid movement, monopodal specimens should be used.

Summary

The rate of locomotion in *Amœba proteus* is highest in monopodal and lowest in multipodal specimens. The average rates obtained for 20 specimens in .001N sodium chloride was 278 micra per minute for monopodal forms, 260 micra per minute for irregular monopodal forms, 125 micra per minute for multipodal forms, and 211 micra per minute for an average of all forms.

The variation in rate is least for monopodal amœbæ and greatest for multipodal amœbæ.

The rate of locomotion of any single individual over a seven-minute period is least variable for monopodal specimens and most variable for multipodal specimens.

The rhythmicity of locomotion in *Amœba proteus* observed by Schwitalla (1924) is largely due to change in form, the rate increasing as the amœba becomes more nearly monopodal and decreasing as it becomes more irregular in form.

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ORDERS OF INSECTS WITH HEART-BEAT REVERSAL

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INTRODUCTION

Intermittent (periodic) reversal of direction of heart-beat, formerly regarded as limited to the Ascidea, has been described by Bethe (1927) as normally occurring in *Phoronis psammophora.*

A similar but transitory intermittent reversal in the embryo before normal direction is established has been observed by Bremer (1931–32) in the chick and by Yokoyama (1929) in the silkworm. In the former it appears to be of rare occurrence; in the latter a characteristic of the embryo anticipating the normal periodic reversal to be seen in the prepupa, pupa, and imago.

In response to experimental conditions, reversal of heart-beat during early development of a teleost (*Macropodus viridi-auratus*) was noted by Gowanloch (1923) and in the chick embryo by Johnstone (1925).

Since the publication of my paper (Gerould, 1929)[1] describing the wide-spread and probably universal occurrence of this phenomenon in Lepidoptera after the larval stage with its forward beating has passed, I have observed it in other orders of holometabolous insects. In none of the orders with complete metamorphosis, except possibly Neuroptera (*Corydalis*) not yet thoroughly examined, have I failed to find examples of it in the imago.

DIPTERA

In a crane fly, *Pachyrhina ferruginea,* peristalsis of the dorsal vessel was observed with extraordinary clearness through the translucent integument of the abdomen. Previous experience with moths had shown that removal of head and appendages does not prevent or seriously affect periodic reversal. The head, wings, and legs were accordingly removed, and peristalsis was observed in abdominal segments 3–5.

I have rarely seen in any insect such a regular series of short alternating phases. In this respect this crane fly resembled the adult

[1] Attention should be called to an error in this paper on p. 426, line 8, referring to pulse rate; "forward" should read "backward." The sentence should read: "It (average pulse rate in forward beating) is roughly twice as fast as in backward beating."

Bombyx mori rather than the pupa or prepupa of that moth. As shown by Table I taken from my first observation (August 16, 1930; temperature 24° C.), the forward phases averaged about 60 beats, the backward about 36; numbers remarkably similar to averages obtained with young silk-moths, which were 51.7 beats forward, 26.5 backward.

The rate of forward beating in the moth was 10 beats in 17 seconds, backward 10 in 29 seconds, but in the crane fly these data show a nearly equal rate in each direction (average 9.9 seconds forward, 9.5 seconds backward). (See Table II). Of especial interest was the regularity with which the rate of backward beating was accelerated during each backward phase, in contrast to the gradual retardation that took place

TABLE I

Heart-beats of the crane fly, Pachyrhina ferruginea (male)

No. Beats in a Phase		No. Beats in a Phase	
Forward Phase	Backward Phase	Forward Phase	Backward Phase
30 + 14 = 44	39	63	35
60 + 16 = 76	38	57	38
53 + 16 = 69	38	63	
64	37	56 + 6 = 62	36
59	34	51 + 5 = 56	36
51		65	34
Av.	60.5 37.2	Av.	60.1 35.8

during the forward phases. Such acceleration was observed in *Bombyx* only during the long backward phase immediately preceding and during pupation.

The regularity in the number of beats in the forward phases in this crane fly was soon interrupted by spontaneous activity of the genitalia, which partially inhibited forward beating and reduced the length of the forward phases from an average of 60 beats to about 30, while the alternate backward phases remained of about the same length as previously.

The same preparation 18 hours later (kept in a moist chamber overnight) showed persistent backward beating, broken at first by pauses into groups of about 36 beats. Over 500 beats were counted, and 37

groups of 10 beats were recorded which showed practically no change in rate (10 beats in 10 seconds).

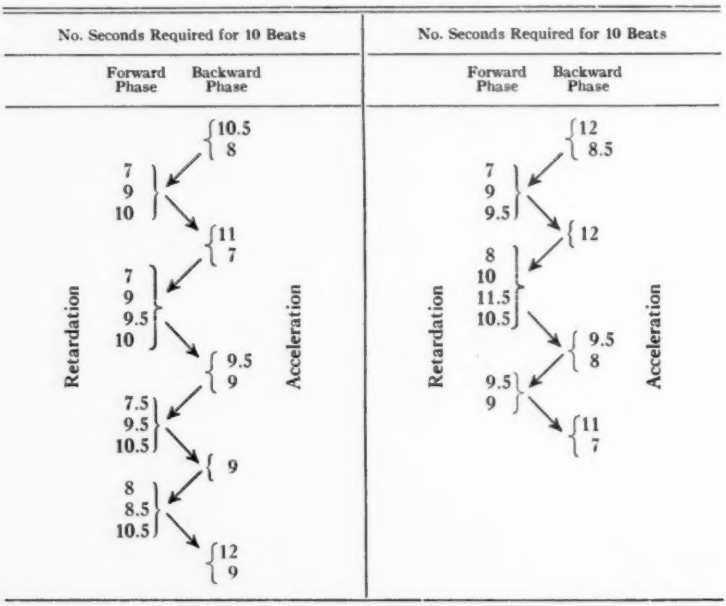
Observations on a drone fly, *Eristalis*, in which beating is extremely rapid, gave among others the following result. Gradual retardation of pulse rate during backward beating is evident. (See Table III).

Coleoptera

In the Coleoptera, no published statement in regard to periodic reversal has come to my attention, though Lasch (1913) in studying the

Table II

Heart-beats of the crane fly (male), Pachyrhina ferruginea. Temperature 22.5° C.



larva of the stag-beetle, *Lucanus cervus*, observed in a few normal larvae a single backward wave extending through a few segments, and in one individual injured by artificial stimulation he saw a succession of backward waves extending through several segments, or merely from one into the next one behind it. His observations, however, were apparently limited to the larva, in which normal periodic reversal is not to be expected.

In August 1931 and again in 1932 I made observations on a large beetle, *Prionus laticollis*. The first individual studied was a ♀ found moribund with leg reflexes still feeble; but, as is generally the case in insects, heart action continued long after leg reflexes had ceased.

It was first observed (4:30 P.M.) by slitting each side of the back and pinning back the body wall so that the ventral surface of the dorsal vessel could be seen; there was vigorous double-action or central beating from the front part of the metathorax, forward through the aorta to the head and backward in the abdominal vessel, in which the rates counted successively were 10 beats in 17, 19, 19, 18, 19, 21, 21, 24 seconds. At 5 P.M. there was again central beating. Counts of the forwardly directed waves in the thorax gave 10 beats in 15, 15, 15 seconds, and during the same phase but backward through the abdomen 10 in 18 seconds (temperature 29° C.).

Kept 18 hours in a moist chamber, the head end of the aorta had

TABLE III

Heart-beats of the drone fly, Eristalis, with abdomen laid open.
Temperature 25° C.

No. Seconds for 10 Beats	No. Seconds for 10 Beats
Forward Phase	Backward Phase
3.5, 3.5, 3, 2.5, 2, 3	4, 4.8, 4.5, 5.5, 5, 5, 6, 5, 5.5, 5, 6, 6.5, 5.8, 6, 6.5, 10, 12.5, 9.5
4, 4, 5.5, 5	

become dry at 10:50 A.M., but backward peristalsis from the anterior part of the metathorax through the abdomen was proceeding at the rate of 10 beats in 21.5, 22 seconds; for a few minutes double or triple beats went backward from the metathorax, but at 11:13 A.M. the groups of two or three had been reduced to single beats with the rate of 10 in 15, 16, 16 seconds. (Temperature 25.5° C.).

In August of the following year, a much longer series of observations was made on a ♂ *Prionus laticollis* which was etherized slightly before the removal of mandibles, antennae, legs, and wings (2:33 P.M., August 6; temperature 29° C.). It was first viewed through the translucent anterior segments of the abdomen. Forward beating at the rate of 10 beats in 9.8, 10.8 seconds was followed by backward, 10 beats in 9, 9 seconds. Then a forward phase was followed by a backward phase of over 300 beats beginning at a rate of 10 beats in 6, 7.25, 7.25 seconds.

The backward beating was slightly more rapid than the forward. After amputation of the head, a series of forward waves began at the rate of 10 beats in 8.6 seconds and ended with 10 in 9.5 and 10 seconds. Air sucked into the dorsal vessel interfered with observations, so a preparation was made of the excised back, showing the ventral surface of the abdominal dorsal vessel, the front of which was inflated with air. Thereupon backward beating started in the inflated anterior part and the following records of the duration of 10 beats were taken: 10 in 9.5, 10, 9, 10.5, 9.5, 10.5, 11, 10.5, 11, 11, 11, 12.25, 12, 12.5, 12, 12.4, 13, 13.5, 14, 15, 14, 14.6, 15.6, 16.5, 16.5, 17, 19 seconds. Beating both ways from the middle of the abdomen followed, freeing the front of the tube of the contained air, the rate of beating steadily falling: 10 in 19, 19.5, 20, 20, 21, 21, 23.5 seconds. The preparation, now drying, was moistened with normal saline solution, and completely forward beating began at a more rapid rate, viz., 10 in 5.5, 7, 8, 9, 9, 8.6, 9.5, 9.5, 9.6, 9 seconds. Then completely backward beating through the whole abdomen ensued; 10 beats in 9.5, 9.5, 9.6, 9.5, 9.5, 9.5, 9.5, 9.6, 9.5, 10, 10, 10, 10.5, 9.6, 10.6, 11, 11.4, 11, 11, 11.25, 11.2, 11, 11, 11, 11, 11.5, 11.5, 11.5, 11.5, 11.5, 12, 12, 12.5, 11.8, 12.5, 12.2, 11.8, 12.2, 12.5, 11.5, 12.2, 11.8 seconds. Then central beating; 10 in 12 (double strokes), 11.5, 11.5, 11, 11, 12 seconds (pausing after the seventh). Then forward altogether: 10 in 12, 12, 11, 12, 11, 11, 11.2, 11.2, 10.5, 11.8, 12, 12, 12, 11.8, 11.2, 11.5, 11.4, 11.2, 11.2, 11.2, 11.6, 12, 12, 11.5, 11.8, 12, 12.75, 11.5, 12.5, (moistened) 11, 11, 13, 12, 12.5, 12, 12.75, 12.2, 12.6, 13, 12.6, 14.6, 16.2, 16.6, 17 seconds. On flooding with saline solution, forward beating continued at increased rate: 10 beats in 7.5, 6.5, 6.5, 7.5, 6.5, 7.2, 7, 7, 8, 8, 8.8, 8.2, 9, 9.5, 9.5, 10, 11, 12, 11.5 + (pause after 7). Reverses, a few beats backward, then forward; pause of 10 seconds, then backward; pause of 17 seconds, then forward, then backward. Length of the observations 2.5 hours (2:33–5:03 P.M.), temperature at the end 29° C. as at the beginning.

The following morning the preparation was alive (in moist air) and a long phase of forward beating observed, the rate of which showed gradual and regular retardation. At first there were pauses between each group of about 10 beats. A succession of counts showed 10 beats (forward) in 5.8, 5.8, 6.5, 6.2, 7.4, 6.6, 6.5, 7.5, 6.8, 6.8, 7, 7.5, 7.5, 7.6, 7.6, 8, 8.5, 7.5, 8, 8, 8, 8.2, 8, 9, 10, 10.5, 11, 10, 10, 10 seconds. There were now less than 10 beats in a phase, and soon groups of 2 beats came; counts of 10 pairs (forward) in 17.5, 19.5, 18, 19, 20, 20, 20, 20, 21, 20.6, 21, 22 seconds. Now single beats replaced pairs and counts of 10 gave 24, 25.4, 24.5, 28, 28, 25.5, 25, 27, 29, 28, 29, 26.5, 29, 33 seconds and beating stopped. Saline solution was applied, and a

series of converging beats conflicting at the third abdominal segment (backward in front of the third segment but still forward behind it) gave the following series of counts: 10 beats in 9.5, 8, 7.5, 8, 7, 8, 7.5, 7.5 seconds.

The same preparation 24 hours after the first observations, temperature 28.5° C., showed a long run of short groups of backward beats separated by pauses of about 10 seconds. There were groups of 7, then of 5 in 3, 4, 4, 3.8, 3.2, 3, 2.5 seconds.

TABLE IV

Heart-beats of solitary wasp, *Sphex*, without head or wings, first three abdominal segments observed. Temperature 28° C. First phase, observed in forward beating, lasted 25 minutes.

Forward Phase	Backward Phase
Rate 10 beats in 2 seconds	10 beats in: 2.6, 2.5, 2, 2.5, 2.5, 2, 2.5, 2.2, 2.6, 2.5, 3, 4, 3, 3.5, 2.6, 2.6, (lags), 5, 4.5, 5, 8, 9 (in flashes of 2), 14, 15, 17, 16, 26, 27.8, 32, 44.5, 83, 82 seconds, length of the phase 32 minutes, last half in groups of 2 with longer and longer pauses between successive pairs.
10 in 11 seconds	
11 seconds	
11 seconds	

HYMENOPTERA

Observations on individuals of this order are less easy and satisfactory than in other groups of higher insects for two reasons, the great length of the phases and rapidity of pulsation. Ants and bees, with their short dorsal vessels with extremely rapid beating, are particularly unfavorable. I have not yet been able to get reliable data on reversal in either group.

In a large solitary wasp, *Sphex*, however, with translucent tergum at the base of the abdomen, satisfactory proof of reversal was obtained, as Table IV shows.

This shows a backward phase of 32 minutes of exceedingly rapid beating, gradually diminishing in rate until at the end of the phase it became very slow.

An ichneumon fly, *Opheltes glaucopterus*, found moribund, reaction of appendages having ceased, gave some excellent data showing unmistakable periodic reversal, with the rapid beating and long phases

characteristic of this order. The heart was observed through the translucent tergites; body walls, head, and appendages were intact. The first observation was of a long forward phase of 27 minutes (11:23 A.M.–12 M., temperature 26.5° C.). Due to the rapid rate, groups of 20, rather than 10 beats were measured; 20 in 15 seconds at first, in 16 seconds at the end (12 readings). A second observation began at 3:20 P.M. with a backward phase of 47 minutes during which the duration of groups of 20 beats remained nearly constant, with very slight acceleration (average of 20 beats in 22.2 seconds for the first 5; 18.9 seconds for the last 5 measurements). At 5–5:08 P.M. measurements of the rate of another clearly backward phase gave an average of 18.5 seconds for 20 beats with no change. (Temperature 26.5° C.).

Orders Probably without Heart-beat Reversal

Examples of adult Neuroptera and Odonata have shown no reversal. A female dobson fly, *Corydalis cornuta,* the large size and comparatively slow pulse of which make it favorable for study, was under observation for 40 minutes; no reversal occurred. One of the Chrysopidae translucent enough for external examination showed no heart-beat reversal.

In a dragon fly, *Plathemis trimaculata,* pulsation of the dorsal vessel, observed in three males and one female, was only forward.

Summary

Representatives of four orders of holometabolous insects, Diptera, Coleoptera, Lepidoptera, and Hymenoptera have shown periodic (intermittent) heart-beat reversal in the pupa and imago. In an adult crane fly, the number of forward beats per phase exceeded the backward, but the average rates of beating in each direction were almost equal. Gradual retardation of rate occurred within each single forward phase, slight acceleration within each backward phase. In the adult beetle there was likewise no marked difference between the average rates backward and forward. A very gradual retardation in rate was observed during each phase whether backward or forward. Central beating in both directions from the pulsating vessel of the metathorax or from the middle of the abdomen occurred. A few converging waves conflicting at the third abdominal segment were observed. Saline solution quickened the rate. There was an occasional tendency to beat in groups of two without change of rate. Pauses sometimes broke up a long phase, each being of about the same length as the groups among which they were interpolated. In Hymenoptera, long backward and forward

phases, each a half hour or more in length, were characteristic of *Sphex* and *Opheltes*. The rates were rapid. In *Sphex* the rate backward was gradually retarded; in *Opheltes* the rate during any long phase was nearly constant, though during one backward phase of 47 minutes there was slight acceleration.

In general, normal reversal occurs independently of the central nervous system and is essentially myogenic. Stimuli from the head, wings, legs, and genitalia, however, secondarily affect peristalsis in detail, producing variations in rate, length of phase, number of beats in a phase, and occasionally probably reversal. Central beating does not depend upon special ganglia but upon local regions of higher irritability (or greater local inflow of haemolymph from the pericardium), which vary somewhat even in the same individual.

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THE MORPHOLOGY OF THE LATER STAGES OF BALANUS CRENATUS BRUGUIERE

LUDWIG E. HERZ

The development of barnacles has occupied the attention of numerous workers since 1830, when J. V. Thompson discovered that nauplii appeared in their development. This similarity in development with Crustacea led to the transfer of the class Cirripedia from the phylum Mollusca, where it had been classified by Linnaeus, Cuvier, and others, to the phylum Arthropoda. In 1843 Burmeister made an important contribution when he found that in *Lepas* the larvæ passed through two totally different stages, the nauplius and the cypris. He had received what he termed a "ball of *Lepas*" on which the cypris stages had just attached and the adults were dismissing the newly hatched nauplii. He thus missed the intermediate stages in the developmental series. In 1875 Dr. R. von Willemoes-Suhm studied the life history of *Lepas fascicularis* while making a cruise from Japan to Sandwich with the Challenger expedition. Concerning this subject he wrote: "My object is to give an idea of the whole development of one Lepas as accurately as possible which seems never to have been done as yet as our knowledge consists of fragments collected mostly in the same way in which Burmeister gained his information." Dr. von Willemoes-Suhm (1875) found that six different nauplius stages and one cypris stage appeared in the development of *Lepas fascicularis,* a pedunculate barnacle of the suborder Lepadomorpha. His report, owing to his untimely death, was published by a co-worker. No mention was made of the transformation of the cypris stage into the adult form. No records of a complete study of the development of an operculate barnacle in the suborder Balanomorpha have been found as yet, nor has any other study of another species of barnacle approached the degree of completeness attained by von Willemoes-Suhm.

This investigation takes up the life history of *Balanus crenatus* Bruguiere of the suborder Balanomorpha, from the newly-hatched nauplius to the adult form. Eight nauplius stages and one cypris stage were found. This study was undertaken upon the suggestion of Dr. Harold Heath of Hopkins Marine Station, Pacific Grove, California, and carried on at Stanford University during the academic years of 1928–29 and

1929–30, and at Hopkins Marine Station during the summer of 1931.[1]

Reasons for the success of this research and the failures of earlier workers may be briefly told. Along the shores of the ocean where many species of barnacle abound and reproduce, it is exceedingly difficult to segregate the nauplii of the different species that are secured by means of plankton hauls since there is a great similarity in their sizes and general structure. The previous investigators therefore attempted to rear in aquaria the newly-hatched nauplii that were taken from the mantle cavity of gravid adult barnacles to secure the complete series of stages in one species, and eliminate the possibility of including the nauplii of other species. This method did not prove successful since the nauplii invariably died after moulting two or three times. The writer met with these same results when efforts were made to isolate and raise the first stages of five of the species of barnacle [2] found on the shores of Monterey Bay. All sorts of containers ranging in size from Syracuse glasses to gallon battery jars in which fresh and balanced or rotted sea water was placed were used as aquaria, but in no case was it possible to secure any moults beyond the third stage. The larvæ would usually remain alive in the third stage for periods of time ranging from eight to twenty-four days. Another method used was to isolate the newly-hatched stages of nauplii in quart jars, cover the jar mouths with cloth, and anchor the jars to floats at various depths, ranging from a few inches to ten feet. The nauplii invariably died in the third stage. These experiments indicated that: (1) the nauplii are very delicate; (2) their mortality rate is very high; (3) their most favorable environment is limited to the open ocean near the shoreline where the water is well aërated by the dashing of the breakers over the rocky shore. Success was attained, however, when experiments were transferred to the hardier specimens of the two species of barnacle that are found in the sloughs of San Francisco Bay. These species are *Balanus crenatus* Bruguiere,[3] and *Balanus glandula* Darwin. *Balanus crenatus* was selected for investigation, as its breeding period extended from March until November, while *Balanus glandula* discontinued breeding after June. Ripe eggs were taken from

[1] The writer wishes to thank Dr. Heath for his direction throughout the work, and wishes to express his appreciation to the following: to Dr. H. A. Pilsbry of the Philadelphia Academy of Natural Sciences for identifying specimens of barnacle; to Professor G. F. Ferris of Stanford University for valued advice; to Dr. W. K. Fisher for the use of the facilities of Hopkins Marine Station; to Dr. Tage Skogsberg for valued advice on drafting the plates of this research.

[2] The five species were *Mitella polymera* Sowerby, *Balanus glandula* Darwin, *Balanus tintinnabulum* Linnæus, *Tetraclita squamosa rubescens* Darwin, and *Lepas Hillii* Leach.

[3] The type locality of *Balanus crenatus* is the coast of England. It is found in the Arctic Ocean; North Atlantic south to Long Island Sound; Bering Sea and North Pacific south to Santa Barbara, California, and Northern Japan.

the mantle cavities of these barnacles and placed in aquaria. After the passage of three days, nauplii in the fourth or fifth stages were found. The later stages were found after the lapse of seven to ten days, and the newly-attached adult form barnacles appeared after thirteen or fourteen days. The times that are required for the development of the later stages of *Balanus crenatus* no doubt vary with the temperature. The times recorded in this paper were secured by observing aquaria specimens that were hatched from ripe eggs. One-gallon battery jars were used as aquaria. These were kept near the windows of a laboratory located on the second floor of the Zoölogy building at Stanford University. A number of thermometer readings taken during July and August, 1930, showed that the temperature of the aquaria did not vary greatly from day to day. The lowest temperature recorded during this time was 17° C., while the highest was 21°. This fluctuation in temperature is perhaps less than that of the slough waters, which are often warmed considerably when the incoming tides flow over exposed and warmed mud flats.

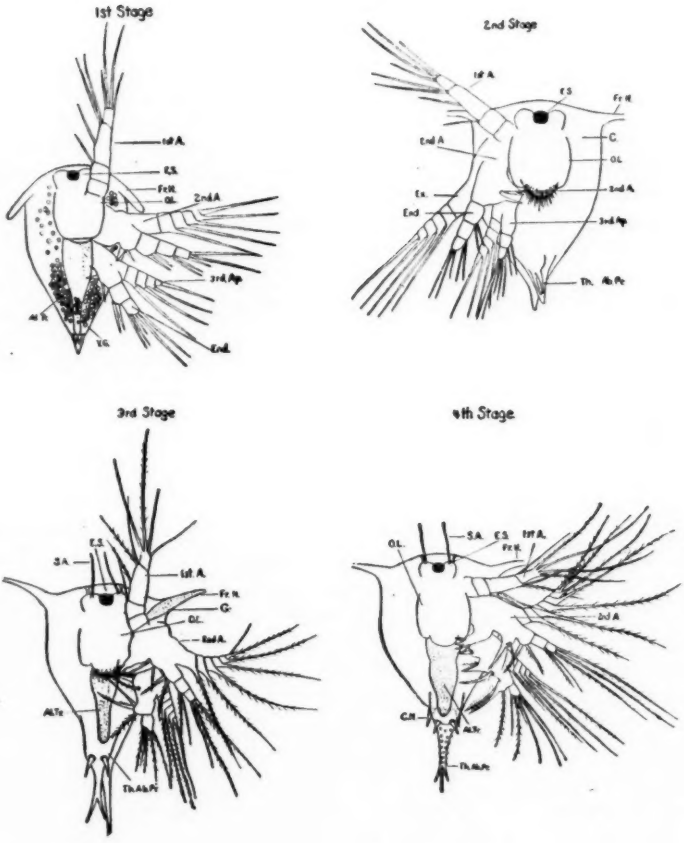
The specimens were easily located, since the nauplii are positively phototropic and the cypris attach on the illuminated side of the aquaria.[4] The nauplii were removed with a pipette to a slide and mounted in glycerine. The cypris stages were mounted in euparal and balsam. Bouin's solution was used as fixing medium. Älum carmine and Delafield's hæmatoxylin were used as stains for the whole mounts of the cypris stage.

The eggs are found in gelatinous masses in the branchial cavity. These are oval, measuring .19 mm. in length and .12 mm. in width. The freshly-spawned eggs are light gray in color. As the eggs ripen, the masses assume a definite shape, and the eyespots and appendages are easily distinguished. The egg membranes are ruptured by the movements of the appendages of the nauplii.

The first stage nauplius measures .22 mm. in length from the anterior portion of the carapace to the tip of the thoraco-abdominal process. It measures .12 mm. in width across the carapace immediately posterior to the frontal horns. An eyespot appears in the mid-section of the anterior portion of the nauplius. It is square in outline, and is situated in the center of a bi-lobed structure. The oral lip is situated immediately posterior to the eyespot. This assists in injestion. The thoraco-abdominal process is quite rudimentary. It projects but slightly beyond the posterior tip of the carapace and ends as a two-pointed structure. The alimentary canal is a simple appearing tubular organ.

[4] This result differs from the findings of Dr. Paul J. Visscher, who reports that the cypris of *Chthamalus fragilis*, *Balanus improvisus*, and *Balanus amphitrite* are negatively phototropic at the time of attachment.

Plate I



1st A., First Antenna
2nd A., Second Antenna
Ex., Exopodite
End., Endopodite
E. S.. Eye Spot
Fr. H., Frontal Horn
C., Carapace
O. L., Oral Lip
3rd Ap., Third Appendage
Th. Ab. Pr., Thoraco-Abdominal Process
Al. Tr., Alimentary Tract
G., Gland
Y. G., Yolk Globule
S. A., Sensory Appendage
C. H., Caudal Horn

The mouth is located at the oral lip and the anus between the two-pointed extremities of the thoraco-abdominal process. The nauplius is propelled by means of three pairs of appendages. The anterior pair is uniramous and the second and third are biramous.

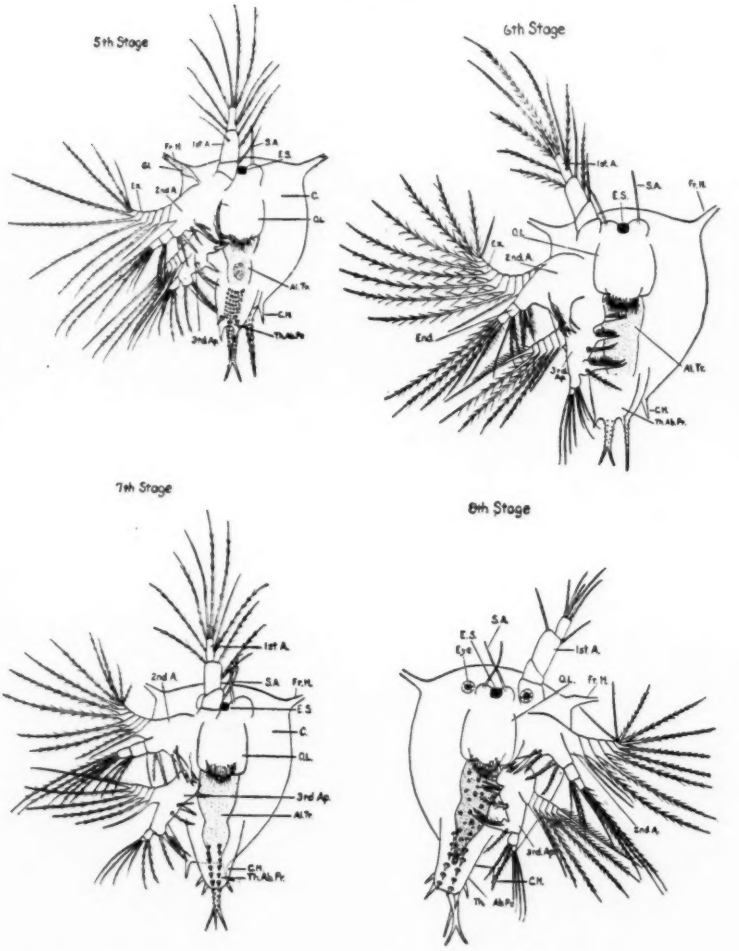
The second stage is slightly larger than the first. It measures .20 mm. in length and .14 mm. in width. The thoraco-abdominal process is larger and more clearly visible. The anterior horns, which pointed in a posterior direction in the first stage, project outward at right angles to the anterior-posterior line. The appendages have no new structures.

The third stage measures .31 mm. from the anterior end of the carapace to the tip of the thoraco-abdominal process. The width of the carapace immediately posterior to the anterior horns is .15 mm. The thoraco-abdominal process has elongated greatly. It is made up of two distinct parts, a longer single-pointed dorsal structure and a forked ventral process. Two stout spines project from the mid-section of the ventral process. The first appendage has no new structures. Setæ have developed on three of its spines. Two new spines are seen on the exopodite of the second appendage, making a total of seven on this ramus. A stout three-pointed, spine-bearing structure appears on the gnathobase. This plays an important part in injestion of food. One new short spine has developed on the exopodite of the third appendage, making a total of five on the ramus; a new spine appears on the second segment of the endopodite of this appendage.

Two large spines have developed at the posterior end of the carapace of the fourth stage nauplius. This stage measures .27 mm. in length from the anterior tip of the carapace to the base of the caudal horns. The anterior horns do not stand out as prominently as in the previous stage. New spines appear on the following: on the proximal portion of the outer side of the distal segment of the first appendage; on the basal segment of the endopodite of the second appendage; a minute spine, which is not visible on all specimens, on the distal segment of the endopodite of the third appendage, making a total of six on this segment.

The length of the fifth stage from the anterior tip of the carapace to the base of the caudal spine is .27 mm. The width posterior to the anterior horns is .22 mm. Three new spines appear on the ventral side of the forked structure of the thoraco-abdominal process anterior to the two that appeared in the fourth stage. The two distal segments of the first appendage have undergone considerable modification. The fourth segment is much narrower than the third. It now bears seven spines. Four spines project from the third segment, the proximal spine of the outer two being not visible on most specimens. New spines appear on both rami of the second appendage. Nine spines, two of which are

PLATE II



1st A., First Antenna
Fr. H., Frontal Horn
GL., Gland
Ex., Exopodite
2nd A., Second Antenna
End., Endopodite
3rd Ap., Third Appendage

S. A., Sensory Appendage
E. S., Eye Spot
C., Carapace
O. L., Oral Lip
Al. Tr., Alimentary Tract
C. H., Caudal Horn
Th. Ab. Pr., Thoraco-Abdominal Process

new, project from the exopodite. Two new spines have developed on the distal segment, and one new spine on the third segment of the endopodite. No new spines appear on the rami of the more conservative third appendage.

The sixth stage nauplius measures .35 mm. in length from the anterior tip of the carapace to the base of the caudal horns and .34 mm. in width. The first appendage has changed little. The exopodite of the second appendage bears eleven spines, an increase of two over the previous stage. One new spine projects from the exopodite of the third appendage, making a total of six on this ramus. Two new spines project from the thoraco-abdominal process external to the three that appeared in the last stage.

The seventh stage measures .46 mm. in length from the anterior tip of the carapace to the base of the caudal horns and .35 mm. in width.

TABLE I

Tabulation of number of spines on first appendage and on exopodites of second and third appendages.

Stage	First Appendage	Ex. Second Appendage	Ex. Third Appendage
1	8	5	4
2	8	5	4
3	8	7	5
4	9	7	5
5	12	9	5
6	11	11	6
7	12	12	6
8	?	12	6

The most important of the new structures apparent in this stage is the anlage of the six pairs of swimming appendages which function in the cypris stage. Six pairs of stout spines have developed on the ventral surface of the thoraco-abdominal process, giving the anlage added protection. The second appendage is the only one that has a new spine. This projects from the exopodite, making a total of twelve on this ramus.

The eighth stage measures .50 mm. in length, and .41 mm. in width. Two eyes have developed lateral to the median eyespot. The undifferentiated anlage which appeared in the thoraco-abdominal process of the previous stage has metamorphosed into rudimentary swimming appendages. These are found within the walls of the thoraco-abdominal process. The three nauplius appendages have, with the exception of the first, undergone no change. The spines of the first appendage have degenerated and become short and stubby.

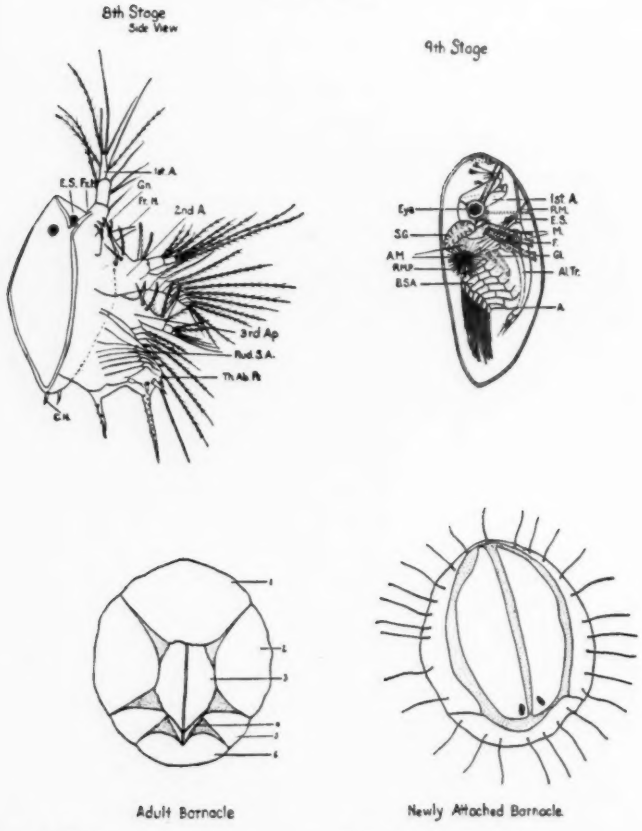
A tabulation of the number of spines on the first appendage and on

the exopodites of the second and third appendages in Table I shows the totals given. An interpretation of the data listed indicates that the number of spines on the appendages of each stage is quite definite and the rate of increase during the development is orderly. The number of spines listed for the first appendage of the fifth stage is open to question, since a minute spine which is not present on all specimens of this stage is included in this total.

The cypris stage measures .55 mm. in length and .24 mm. in width. Its valves have been formed through a folding of the carapace of the nauplius stage. The first appendage has become modified and now is used as an adhesive organ. The two biramous swimming appendages of the nauplius stages have disappeared. Their rudiments appear to be formed into the mandibles and maxillas in the metamorphosis into the adult form barnacle. Two long slender organs extend ventralwards from the region of the eyes. These are .13 mm. in length and are the sensory appendages of the nauplius stages. These are moved back and forth as the cypris moves along and appear to act as feelers. Four eyespots appear in the forward end of this stage. Two of these are located along the median line of the body on the walls of the valves. These quiver constantly during the movements of the cypris. Two smaller eyespots are present dorsal to these two. The larger eyespots and the first antennæ are attached to the valves by some eight or ten muscular strands. The abdomen, bearing the six pairs of propelling appendages, is a very flexible organ. It moves on a pivot formed by a set of muscles which attach the abdomen to the valves near the mid-ventral portion of the body. The abdomen is capable of being completely withdrawn within the protective walls of the valves during quiescent periods; in times of movement the abdomen turns ventrally, placing the appendages in position for vigorous movement, which causes the cypris to dart ahead at a rapid rate. The abdomen is prevented from turning outward too far by a stout muscle (R. M. of ninth stage on Plate III) that connects the abdomen with the dorsal walls of the valves near the anterior end of the cypris. An enlarged area of the enteric tract that may be termed the stomach appears just posterior to the insertion of the dorsal retractor muscular strand. The enteric tract passes posteriorly and the anus appears between the sixth swimming and the seventh rudimentary appendages. A pair of shell glands are located anteriorly to the adductor muscles. A small duct passes dorsally from the gland. Its subsequent course was not definitely determined.

The cypris stage, when on the point of transforming into the adult form, settles on some hard surface. The first antennæ are extended

PLATE III



E. S., Eye Spot
C. H., Caudal Horn
Fr. H., Frontal Horn
1st A., First Antenna
Gn., Gnathobase
2nd A., Second Antenna
3rd Ap., Third Appendage
Rud. S. A., Rudimentary Swimming Appendages
Th. Ab. Pr., Thoraco-Abdominal Process
S. G., Shell Gland

A. M., Adductor Muscle
R. M. P., Rudimentary Mouth Parts
B. S. A., Biramous Swimming Appendages
1st A., First Antenna
R. M., Retractor Muscle
E. S., Eye Spot
M., Muscle
F., Food
Gl., Gland
Al. Tr., Alimentary Tract
Anus, Anus

far out from the valves and the two pairs of eyespots are found near the ventral portion of the body. The muscles and ligaments that attach these parts to the valves are extended to their fullest extent. These are eventually ruptured and the antennæ and the larger of the two pairs of eyespots are cast off. The remaining portions of the cypris are loosened from the valves by the kicking action of the appendages, and these parts slip out from the old chitinous layer that surrounds them. A gelatinous substance exudes from the body membrane at the base or point of attachment. This develops into the compartments. The number of the compartments is not clearly visible when they are first layed down, but they become more distinct as the barnacle grows. The plates merge at the base and form a thin calcareous layer which lines the base of the barnacle. The scuta and terga develop immediately above the gelatinous matrix of the compartments. In the adult barnacle the scuta and terga are considerably below the tips of the six compartments. This appears to be due to the upward growth of the compartments, rather than to an invagination of the membrane from which the terga and scuta develop. The rapidly-moving swimming appendages of the cypris stage have transformed into slow-moving food-securing organs.

Conclusion

Balanus crenatus Bruguiere passes through eight nauplius stages and one cypris stage in its development from the egg to the adult form. The time necessary for this to take place is from two to three weeks.

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